Casein - whey protein interactions in heated milk

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Caseïne - wei-eiwit interacties in verhitte melk

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Abbreviation key

α-lac	α-lactalbumin
β-lg	β-lactoglobulin
CE	capillary electrophoresis
CL	cross-linked
CMP	casein macropeptide
DTT	dithiothreithol
DWS	diffusing wave spectroscopy
GDL	glucono-δ-lactone
NEM	N-ethylmaleimide
pH-T route	increasing temperature at a constant pH
pI	iso-electric pH
RCT	rennet clotting time
SDS	sodium dodecyl sulphate
TCA	trichloro-acetic acid
T-pH route	decreasing pH at a constant temperature
WPF-milk	whey-protein-free milk

Chapter 1: Introduction

Milk

Milk and milk products have been consumed by people all over the world throughout history. Milk is the secretion of the mammary glands of mammals and is intended for nutrition of the neonate. However the milk of cow, buffalo, sheep and goat, and on a smaller scale also milk of camel, lame and horse, is also used by man as a nutritional source. This thesis focuses on bovine milk produced by domesticated cows, which will be referred to as milk throughout this thesis.

In the Netherlands about 11 billion kg of milk was produced in the year 2000. In the European Union this was 114 billion kg and worldwide at least 280 billion kg (35). In the Netherlands about 10% of all the milk is consumed as pasteurised, sterilised or UHT milk. The major amount of the milk is not consumed directly, but is further processed to obtain products like cheese, butter, milk powder, condensed milk, quark and yoghurt. About 50% of the milk is used for the production of cheese and 5% for the production of yoghurt. (35)

For microbiological safety all the milk is heat treated before use, but that also changes the functional properties of the products. Different temperature treatments are required for the different products that can be made from milk. Examples are thermisation (e.g. a few seconds at 65°C), which eliminates most psychrotrophic bacteria; these bacteria can produce very heat-resistant lipases and proteinases, and their inactivation prevents deterioriation of the milk during storage. Low pasteurisation, heated for e.g. 15 seconds at 72°C, is a treatment that eliminates most of the vegetative microorganisms, but few other permanent changes are induced. This milk is used for the manufacturing of cheese. High-pasteurised milk (e.g. 20 seconds at 85°C) is used for the production of yoghurt and Ultra High Temperature (e.g. 1 second at 145°C) for preparation of UHT-milk (41, 46).

From a macroscopic point of view milk is a white opaque liquid with a Newtonian viscosity of 1.5 times that of water. On standing a typical cream layer develops, which may be redispersed by gentle stirring. As a colloidal dispersion milk is extremely stable. It can be boiled, frozen or dried and redispersed without any obvious changes in stability. The stability is the more remarkable as milk is a mixed colloidal dispersion. It contains fat globules about 1μm in radius,

casein micelles of 0.1μm and whey proteins of 0.003μm, and numerous small molecules of less than 0.001μm.

In the Netherlands the fat globules constitute about 4.4 % (w/w) of the milk. They consist of numerous different lipids of which most are made up of triglycerides.

The casein micelles constitute about 2.8% (w/w) of the milk or 3.05% including calcium phosphate. The micelles are roughly spherical particles. The different caseins in the micelle are classified as proteins that are insoluble at pH 4-5.

Whey proteins constitute about 0.5% (w/w) of milk and are the proteins that remain in solution after coagulation of the casein micelles due to rennet treatment as used for cheese manufacturing. Another definition is that they do not precipitate in the pH region 4-5.

The numerous small particles present in milk are mainly lactose and salts. Lactose is the major sugar in milk (4.6% (w/w) average). It is a reducing disaccharide composed of glucose and galactose. It gives milk a slightly sweet taste and it is the principal carbon source for most of the microorganisms that grow in milk. The main salt ions present in milk are potassium, sodium, magnesium, chloride and phosphate. (46)

Casein micelles

Casein micelles are association colloids consisting of different caseins, i.e. α_{s1}-, α_{s2}-, κ- and β-casein, with an average diameter of 200 nm. The four caseins have different amino acid sequences and they differ greatly in charge distribution and in tendency to aggregate in the presence of Ca^{2+} ions (38, 46). The α_{s1}-, α_{s2}- and β-caseins are all calcium sensitive and they would precipitate at calcium concentrations as present in milk. In contrast κ-casein is insensitive to calcium and if present in sufficient amount it will stabilise the other caseins against precipitation (22). Most of the calcium-sensitive caseins are located in the interior of the micelle, whereas κ-casein is located at the exterior. The C-terminal part of κ-casein is very hydrophilic and has a considerable negative charge. This part of the molecule sticks out of the micelle into the surrounding medium as a flexible hair, forming a hairy brush at the surface (46). The caseins in the micelles are linked by calcium phosphate nanoclusters. A schematic representation of a casein micelle is depicted in Figure 1.1 .

Dissolving the calcium phosphate, e.g. by addition of EDTA, causes a loss of integrity of the micelle. After some time the casein micelle dissociates and is invisible by electron microscopy. The important role of calcium phosphate in maintaining integrity of the casein micelles finds its origin in its biological function, which is to transport calcium to the neonate and to prevent calcinations of the mammary gland. Calcium phosphate which is present in the surrounding medium is regarded as dissolved or serum calcium phosphate. Serum and micellar calcium phosphate are in an equilibrium which is shifted mainly by pH. No effect of temperature variation on the dissociation was observed in the range of 4 to 30°C (5). Additionally, part of the casein is not present in the micelle but in the surrounding medium as serum casein. By definition serum caseins are the caseins which remain in solution after precipitation of the casein micelles by ultracentrifugation. Colloidal and serum casein are in an equilibrium which is dependent on pH and temperature. Lowering the temperature and/or pH increases the amount of serum casein. Increasing the temperature has just the opposite effect. The pH at which maximum dissociation occurs shifts to higher pH values at higher temperatures (6).

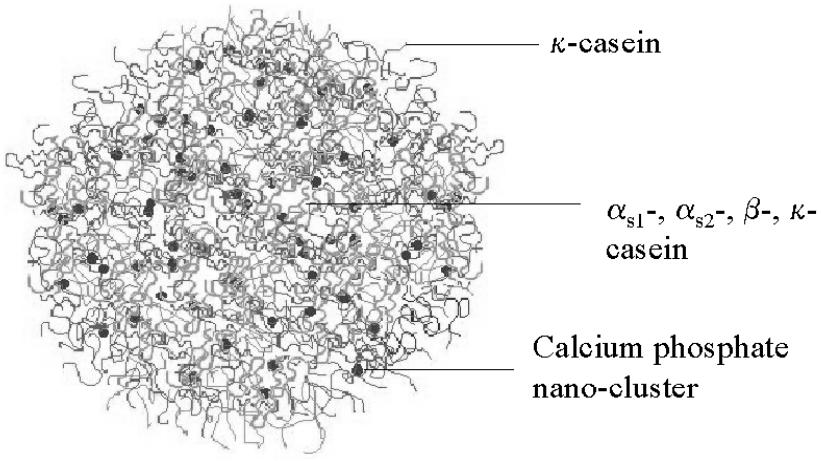

Figure 1.1: A schematic representation of a casein micelle consisting of four different caseins, i.e. α_{s1}-, α_{s2}-, β- and κ-casein, linked together by calcium phosphate clusters. κ-Casein is mainly present at the exterior of the casein micelle forming a “hairy brush”. This figure is taken with permission from Holt (51).

Whey proteins

When milk at room temperature, is brought to pH 4.6, the caseins precipitate. The supernatant contains several proteins that are collectively called whey proteins. Historically, whey proteins are the proteins present in the whey of cheesemaking, including the caseinomacropeptide; here we apply the more general definition of non-casein proteins in milk. Most of the whey proteins are globular proteins. They include β-lactoglobulin (β-lg), α-lactalbumin (α-lac), bovine serum albumin (BSA), immunoglobulins (Ig) and several minor proteins and enzymes. Their concentrations are respectively 3.2, 1.2, 0.4. 0.75, 0.6 g/l. This study focuses on β-lg and α-lac as the major whey proteins in milk. Upon heat treatment they denature and aggregate and they determine the properties of heated milk.

β-lactoglobulin (β-lg)

β-lg binds retinol and its proposed biological function is to transfer the vitamin from maternal milk to the neonate via specific receptors in the intestine. In milk six genetic variants of β-lg are known, of which the A and B variants are the most common and occur with an almost equal frequency. In this thesis no distinction is made between the different genetic variants. The iso-electric point of the protein is 5.1. At room temperature and physiological pH (pH 6.7) β-lg exists mainly as a dimer, but it dissociates into monomers at higher temperatures. X-ray measurements have indicated that the dimer is approximately an ellipsoid of length 6.45 nm and width 3.6 nm. This dimer is composed of two impinging spheres of 3.6 nm diameter that are non-covalently bound. The β-lg monomer has a molecular mass of 18.3 kDa and comprises 162 amino acids. The β-lg monomer contains two disulfide bridges (Cys106-Cys119 and Cys66-Cys160) and one free cysteine (Cys121). The secondary structure consists of approximately 15% α-helix and 50% β-sheet, the rest being unordered structure. The tertiary structure of β-lg consists of nine strands of antiparallel β-sheet of which eight are wrapped round to form a flattened cone or calyx. The ninth β-strand is at the dimer interface and forms an anti-parallel β-sheet interaction with the associated monomer. The free thiol group, Cys121, and one of the two disulfide bridges (Cys106-Cys119) are buried at the sheet-helix interface. The other disulfide bond (Cys 66-Cys160) is on the outer

surface in a mobile part of the molecule close to the C-terminal region (15, 44).

α-lactalbumin (α-lac)

The biological activity of α-lac is to promote the binding of glucose to galactosyltransferase, which enhances the transfer of galactose from UDPgalactose to glucose, an essential step in the formation of lactose. α-lac in milk is a compact, low molecular mass (14.2 kDa) globular protein which is well characterised. Three genetic variants are known, respectively A, B and C. In milk of Western breeds only the B variant is found. It consists of 123 amino acids with four disulfide bridges (Cys 6-120, 28-111, 61-77, 73-91). The iso-electric point is 4.2-4.5. Circulair Dichroism spectra indicate that at physiological pH, the secondary structure of α-lac shows 26% α-helix, 14% β-sheet and 60% unordered structure. α-lac is a calcium-binding metalloprotein which is also capable of binding zinc and probably other metals (44, 46).

Heat-induced interactions between casein micelles and whey proteins

The main reason for heat treatment of milk is to improve its keeping quality by destroying microorganisms and to achieve desirable properties in the final product (41). Upon heat treatment of milk several processes take place. The most obvious effect is the denaturation of whey proteins. The whey proteins undergo conformational changes, which in principle is a reversible process. However in practice this is only true for pure α-lac solutions. In the case of β-lg the conformational change of the molecule results in the exposure of a reactive thiol group. This reactive thiol group can form disulfide links with other reactive thiol groups and through thiol group-disulfide bridge exchange reactions. This process resembles a polymerisation process, in which the unfolding step of β-lg represents the initiation (20, 37). The differences between the whey proteins upon heating are caused by the fact that α-lac contains only disulfide bridges, while β-lg has a free thiol group in addition to two disulfide bridges. Therefore α-lac, which can renature completely when heated alone, is also irreversibly denatured in the presence of β-lg due to thiol group-disulfide bond exchange reactions. Heat treatment of milk

results in the formation of whey protein aggregates containing both α-lac and β-lg.

No noticeable effects are observed on the casein micelle fraction due to heat treatment in the temperature range 70-100°C (23). The caseins have a mainly random coil structure and are therefore not susceptible to denaturation processes. Heat treatment also causes precipitation of serum calcium phosphate. After cooling, the precipitates dissolve again, but this is a slow process. Although calcium phosphate is an important component of casein micelles the stability of milk itself is not severely influenced by this precipitation.

If both whey proteins and casein micelles are present, as in milk, interactions between the two groups of protein also occur. Through thiol group-disulfide bond exchange reactions the whey proteins can interact with κ-casein present at the exterior of the casein micelle. The initial step of this process is believed to be physical in nature, but the final interaction is often covalent, i.e. a disulfide linkage (13, 19). This results in casein micelles stabilized by a hairy brush of κ-caseins with mainly covalently associated whey proteins. As in pure whey protein solutions also in this case β-lg acts as a crosslinker between casein micelles and α-lac.

Heat treatment of milk results in a complex mixture of native whey proteins, whey protein aggregates and whey protein coated casein micelles. Research on heat treatment of milk showed that the final composition of the mixture of whey proteins and casein micelles depends on the pH and temperature of heat treatment (4, 6, 23, 25, 33, 34). Both parameters are varied in practice and are therefore very relevant in order to control and predict processes. The pH of milk varies in the range 6.55 to 6.98 during a milking season (32, 49). Also the age of the milk can affect the pH. Microbial and enzymatic activity will lower the pH. Research where either changes in the milk composition were made or model systems were used revealed that salt concentration and composition, the ratio between casein micelles and whey proteins and total protein concentration are also determining denaturation kinetics (1, 2, 4, 8, 24, 25, 43).

Heat treatment of milk is a standard procedure preceding further processing. It is crucial to control and understand the effects of temperature, pH and composition of the milk during heat treatment, as these will determine the characteristics of the final dairy products. Although, as stated above, considerable work has been done on whey protein denaturation due to heat treatment of

milk, many questions are still unanswered. Quantification of the complex mixture of denatured whey proteins and casein micelles in heated milk is still a rather unexplored area. Fractionation techniques have hardly developed in recent years and are not very suitable for unravelling the mixture of whey proteins associated with the casein micelle and whey protein present in soluble aggregates. Therefore, the formation of aggregates is still hardly acknowledged and the aggregates have not been characterised as regards basic properties like composition and size. The distribution of whey proteins on the surface of the casein micelle and the influence of heating conditions, like that of temperature, pH, salt, concentration and composition, is still unknown.

Processing of milk

Casein micelles in milk are stable at the pH of milk, i.e. 6.7, due to the stabilising hairy brush of κ-casein. Also heat-induced changes do not affect the stability of milk at ambient temperatures. However, pH, renneting, salt, ethanol and other additives do affect the hairy brush and are therefore able to destabilise the micelle, causing aggregation and gelation. In the dairy industry gel formation is commonly induced by acidification and renneting in order to obtain milk products like yoghurt and cheese.

Yoghurt is prepared from heat-treated milk, generally high-pasteurised milk. Addition of microorganisms, mainly lactic acid bacteria, starts the fermentation. Lactose, which is the principal carbon source present in milk, is converted into lactic acid. This causes a gradual decrease of the pH and ultimately leads to gel formation. Heat treatment of the milk is of great importance for the quality of the yoghurt. Yoghurt prepared from high-pasteurised milk has a smaller tendency to synerese and has a higher viscosity. Important for the viscosity and flow properties of the yoghurt is the production of EPS (extracellular polysaccharides). These are polysaccharides excreted into the surrounding medium by some species of lactic acid bacteria. They add to an increased viscosity, a decreased susceptibility to syneresis and an increased ropiness of the yoghurt (30).

Preparation of cheese requires a combination of acidification and enzymatic hydrolysis of proteins present in milk. Low pasteurised milk is inoculated with starter culture and rennet and a

gel is formed. The gel is cut, which releases the whey. Part of the whey is removed. Water is added to the remainings, called curd, to remove excess lactose and finally all whey is removed. The curd is put into cheese moulds and pressed. This is followed by brining and finally storage at constant conditions (e.g. 13°C, 88% relative humidity). The fermentation by the starter culture results in a final pH of about 5.3.

This thesis will focus on acidification and renneting of milk, as these processes are the basis for yoghurt and cheese production.

Acidification of milk

The stability of the κ-casein brush remains intact to about pH 5 (at 20°C). Then over a small range of pH the brush collapses; the casein micelles are no longer stabilised and they start to aggregate and gel (22). Both the speed of acidification and the temperature during acidification are important process parameters which can influence the final gel product. In case the speed of acidification is very high, as during addition of HCl at room temperature, flocculation occurs. The casein micelles aggregate very fast and sediment, leaving a clear layer of serum. If acidification is carried out with a starter culture or with a chemical like glucono-δ-lactone, the acidification takes place more slowly and a homogeneous gel is formed. Increasing the acidification temperature causes a shift in gelation pH to higher pH values (27). Additionally higher temperatures decrease the gel strength and increase the permeability of the final gel (3, 27, 28).

Heat treatment of the milk is an important processing step during the manufacturing of yoghurt. As the whey proteins partially associate with the κ-casein, the stability of the brush is reduced remarkably. Heat treatment induces a shift in gelation point to higher pH values (12, 18, 26). The pH at which casein micelles tend to neutrality is increased due to the higher iso-electric point of β-lg (i.e. 5.2) which has associated with the casein micelle upon heat treatment. Also the gel properties are changed significantly by heat treatment of the milk. Syneresis decreases rapidly while the firmness of the gels, measured by resistance to penetration, increases as a function of the percentage of denatured whey protein (9, 10). Also the storage modulus (G') increases due to heat treatment of the milk (26, 29). In contrast the permeability is hardly affected (45). The

effects of whey protein denaturation on the gel properties can be ascribed to several factors. 1] The concentration of gelling protein increases due to the contribution of the denatured whey proteins in the gel structure (2.8% in unheated milk versus 3.3% in heated milk). 2] Denatured whey proteins associated with the casein micelle could act as a bridging material between the micelles. As denatured whey proteins contain reactive thiol groups, disulfide interactions can also occur. This would increase the number and strength of bonds between the protein particles. 3] Whey protein aggregates can act as an additional bridging material.

Heat treatment of milk and the concomitant denaturation of whey proteins affects the characteristics of the acid-gel. Although the role of denatured whey proteins is generally acknowledged hardly anything is known about the role of the different fractions, i.e. whey protein aggregates and whey proteins coating the casein micelle. Also the formation of disulfide links during gelation at ambient temperatures and the role of the different fractions has not been investigated.

Renneting of milk

Chymosin, the major enzyme present in calf rennet as used for cheese manufacturing, acts very specifically on the stabilising hairy brush of κ-casein. This enzyme is collected as a precursor, called prochymosin, from the stomachs of young calves. In an acid environment a peptide is split off releasing the enzyme chymosin. The enzyme is an endopeptidase, which in milk of pH 6.7 cleaves very specifically the Phe105-Met106 bond of κ-casein. κ-casein is split into para-κ-casein and caseinomacropeptide (CMP) of which para-κ-casein is insoluble, while CMP is soluble. Para-κ-casein has not the colloid-protective property of κ-casein. Extensive cleavage of the κ-caseins present in the hairy brush will therefore result in destabilisation of the micelle. The micelles will clot together and form a gel. This is what happens in the manufacturing of cheese (7, 21, 46).

The action of chymosin on casein micelles in milk can be divided into two processes. The first is the enzymatic cleavage and the second is the clotting process. Manufacturing of cheese is preferably carried out with unheated or low pasteurised milk. Under these conditions the action of the chymosin is well investigated. The activity of the enzyme depends on pH, temperature and calcium concentration. For an overview of the effects of these parameters on

enzymatic cleavage, clotting, renneting time and syneresis see Walstra and Jenness (46).

Chymosin acts only on the casein micelles, which means that whey proteins are not included in the curd. For a long time this has been seen as a waste of protein. The easiest solution to include the whey proteins in the cheese is by giving the milk a heat treatment. The whey proteins associated with the casein micelles will become part of the curd. However heat treatment of the milk causes impaired clotting properties and a weaker curd. Furthermore, and importantly, it leads to off flavour in the cheese. For this reason heat-treated milk is less suitable for cheese manufacturing (42, 47).

Whey protein denaturation could affect both steps important for proper curd formation: the enzymatic cleavage of the κ-casein and the subsequent clotting of the micelles. Most publications on this subject indicate that the enzymatic cleavage is inhibited by the reaction of β-lg with κ-casein due to steric hindrance (14, 36, 40, 48, 50), however there are some publications where this in contradicted (17, 31). The second step in the action of chymosin is the clotting of the micelles. In the literature the following explanations are given for the impaired clotting properties of heated milk: 1] Due to incomplete enzymatic hydrolysis, caused by the interaction of β-lg with κ-casein, the micelles remain “fairly” stable (46). 2] Due to heating, calcium phosphate precipitates. This process is reversible, but it takes hours before the original calcium concentration is restored. Therefore, heat treatment causes a temporarily decrease in the serum calcium concentration, at which calcium concentrations destabilised casein micelles are less sensitive to precipitation (16, 39). 3] Association of the whey proteins with the casein micelle stabilises the casein micelle after CMP is released. At the pH of renneting, i.e. around 6.5, whey proteins are still highly charged and will not aggregate spontaneously.

Many simultaneous effects seem to cause the impaired clotting properties of heated milk. However, a number of questions are still unanswered. Different methods have been used to study enzymatic cleavage of the κ-casein and different degrees of inhibition are observed. Therefore, it is unclear whether the methods applied are causing these differences or whether a real inhibition of enzymatic cleavage is caused by heat treatment of the milk. Calcium phosphate seems to play a role but this has only been studied at ultra high temperatures (> 100°C) and no direct evidence of the crucial role of

denatured whey proteins on the impaired clotting properties has been obtained.

Outline of this thesis

This thesis focusses on the stability of casein micelles in heated milk towards aggregation and gelation induced by acidification and renneting. Two types of milk are used throughout this thesis, i.e. reconstituted skim milk (chapter 2, 6 and 7) and fresh skim milk (chapter 3, 4, 5 and 7). In the various chapters both are referred to as milk unless otherwise required. Acidification and renneting are the basis of yoghurt, quark and cheese production. To simplify the systems, yoghurt was mimicked by chemically acidifying skim milk, and cheese was mimicked by renneting milk at its natural pH without using starter cultures. To study the effect of heat treatment of the milk on these gelation processes unheated milk was compared to milk heated at temperatures of 70 to 90°C. To study the role of the different whey proteins and the effects of aggregates and coating on the gelation process, reconstituted whey-protein-free milk was used, so that the whey proteins could be added in any concentration desirable.

Chapter 2 deals with the effect of pH and temperature on the acid-induced gelation of unheated milk. Gelation was induced by varying the sequence of temperature increase and pH decrease. The role of serum casein is related to the gelation point and the visual appearances of the gels obtained.

In chapter 3 the heat-induced denaturation of whey proteins in milk is investigated by developing methods to quantify the amount of denatured whey proteins present in soluble aggregates and associated with the casein micelle. The composition and size of the aggregates are determined.

In chapter 4 the methods developed in chapter 3 are used to investigate the effects of the pH at which heat treatment is performed (6.3-6.9) on the denaturation processes in milk. The composition of the heated milks is related to acid- and rennet-induced gelation.

In chapter 5 a thorough study is described of the effect of heat treatment on enzymatic cleavage by rennet and the impaired rennet-induced clotting and gel forming properties. Several methods for studying the enzymatic cleavage are compared. By using whey-

protein- free milk the heat-induced effects of calcium phosphate precipitation and whey protein denaturation on the impaired clotting properties can be studied separately.

Chapter 6 deals with the acid-induced gelation of heated milk. The observed shift in gelation pH is related to whey protein denaturation. Additionally, the contribution of the two major whey proteins, α-lac and β-lg, to this shift in gelation pH is discussed.

In chapter 7 the type and strength of the bonds formed in gels made from heated milk are compared to those formed in unheated milk gels. Disulfide bridge formation in acid gels is monitored as function of time and formation of disulfide-linked structures is demonstrated.

Summarising, the first part of this thesis (chapters 2-4) describes pH- and temperature- induced changes in milk. This results in two models, one for temperature-induced changes below the denaturation temperature (chapter 2) and one for temperatures above the denaturation temperature (chapter 4). The latter model forms the basis for relating the distribution and aggregation state of the whey proteins to the observed properties of heated milk subjected to gelation (chapters 4-7). In chapter 8 the practical relevance of this work is discussed.

References

1. Anema, S.G. 2000. Effect of milk concentration on the irreversible thermal denaturation and disulfide aggregation of β-lactoglobulin. J. Agric. Food Chem, 48: 4168.
2. Anema, S.G. 2001. Kinetics of the irreversible thermal denaturation and disulfide aggregation of α-lactalbumin in milk samples of various concentrations. J. Food Sci. 66: 1: 2.
3. Arshad, M., M. Paulsson, and P. Djemek. 1993. Rheology of buildup, breakdown, and rebodying of acid casein gels. J. Dairy Sci. 76: 3310.
4. Corredig, M. and D.G. Dalgleish. 1996. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Res. Int. 29: 1: 49.
5. Dalgleish, D.G. and A.J.R. Law. 1988. pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. J. Dairy Res. 55: 529.
6. Dannenberg, F. and H.G. Kessler. 1988. Reaction kinetics of the denaturation of whey proteins in milk. J. Food Sci.53: 258.

7. Dalgleish, D.G. and A.J.R. Law. 1989. pH-induced dissociation of bovine casein micelles II. Mineral solubilization and its relation to casein release. J. Dairy Res. 56: 727.
8. Dalgleish, D.G. 1990. The enzymatic coagulation of milk. Page 579 in Advanced Dairy Chemistry-1: proteins. P.F. Fox, ed. London Elsevier Science Publishers Ltd.
9. Dalgleish, D.G., L. van Mourik, M. Corredig. 1997. Heat-induced interactions of whey proteins and casein micelles with different concentrations of α-lactalbumin and β-lactoglobulin. J. Agric. Food Chem. 45: 4806.
10. Dannenberg, H. and H.G. Kessler. 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 1. Syneresis. Milchwissenschaft. 43: 632.
11. Dannenberg, F. and H.G. Kessler 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 2. Firmness and flow properties. Milchwissenschaft. 43: 700.
12. Heertje, I., J. Visser and P. Smits. 1985. Structure formation in acid milk gels. Food Microstructure. 4: 267.
13. Hill, A.R. 1989. The β-lactoglobulin-k-casein complex. Can. Inst. Food Sci. Technol. J. 22: 2: 120.
14. Hindle, E.J. and J.V. Wheelock. 1970. The primary phase of rennin action in heat sterilised milk. J.Dairy Res. 37: 389.
15. Hoffmann, M.A.M. β-lactoglobulin: denaturation and aggregation. Ph.D. Diss., University of Utrecht, Utrecht, Neth.
16. van Hooydonk, A.C.M, H.G. Hagedoorn and I.J. Boerrigter. 1986. The effect of various cations on the renneting of milk. Neth. Milk Dairy J. 40: 369.
17. van Hooydonk, A.C.M., P.G. de Koster and I.J. Boerrigter. 1987. The renneting properties of heated milk. Neth.Milk Dairy J. 41: 3.
18. Horne, D.S. and C.M. Davidson. 1993. Influence of heat treatment on gel formation in acidified milks. Protein and fat globule modification, in Proceedings of IDF Seminar. 267.
19. Jang, H.D. and H.E. Swaisgood. 1990. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. J. Dairy Sci. 73: 900.
20. de Kruif, C.G., M.A.M. Hoffmann, M.E. van Marle, P.J.J.M. van Mil, S.P.F.M. Roefs, M. Verheul and P. Zoon. 1995. Gelation of proteins from milk. Faraday Discuss. 101: 185.
21. de Kruif, C.G. and E.B. Zhulina. 1996. κ-Casein as a polyelectrolyte brush on the surface of casein micelles. Colloids and Surfaces A. 117: 151.
22. de Kruif, C.G. and C. Holt. Casein micelle structure, functions and interactions.
to be published as Chapter 3 in Advances in Dairy Chemistry. 2002. ed. P.F. Fox and P.L.H. McSweeney.
23. Law, A.J.R., D.S. Horne, J.M. Banks and J. Leaver. 1994. Heat-induced changes in the whey proteins and caseins. Milchwissenschaft. 49: 3: 125.
24. Law, A.J.R. and J. Leaver. 1997. Effect of milk protein concentration on the rates of thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 45: 4255.

25. Law, A.J.R. and J. Leaver. 2000. Effect of pH on the thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 48: 672.
26. Lucey, J.A., C. Tet Teo, P.A. Munro and H. Singh. 1997. Rheological properties at smal1 (dynamic) and large (yield) deformations of acid gels made from heated milk. J. Dairy Res. 64: 591.
27. Lucey, J.A., T. van Vliet, K. Grolle, T. Geurts and P. Walstra. 1997. Properties of acid casein gels made by acidification with GDL 1: rheological properties. Int. Dairy J. 7: 381.
28. Lucey, J.A., T. van Vliet, K. Grolle, T. Geurts, P. Walstra. 1997. Properties of acid casein gels made by acidification with GDL 2: syneresis, permeability and microstructural properties. Int. Dairy J. 7: 389
29. Lucey, J.A., P.A. Munro and H. Singh. 1999. Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. Int. Dairy J. 9: 275.
30. van Marle, M.E. 1998. Structure and rheological properties of yoghurt gels and stirred yoghurts. Ph.D. Diss., University of Twente, Enschede, Neth.
31. Marshall, R.J. 1986. Increasing cheese yields by high heat treatment of milk. J. Dairy Res. 53: 313.
32. Newstead, D.F., W.B. Sanderson and E.F. Conaghan. 1977. Effects of whey protein concentration and heat treatment on the heat stability of concentrated and unconcentrated milk, N. Z. J. Dairy Res. Tech. 12: 29.
33. Noh, B., T. Richardson and L.K. Creamer. 1989. Radiolabelling study of the heat-induced interactions between α-lactalbumin, β-lactoglobulin and κ-casein in milk and in buffer solutions. J. Food Sci. 54: 4: 889.
34. Oldfield, D.J., H. Singh, M.W. Taylor, K.N. Pearce. 2000. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk, Int. Dairy J. 10: 509.
35. Produktschap zuivel. Statistisch jaarboek. overzicht 2000
36. Reddy, I.M. and J.E. Kinsella. 1990. Interaction of β-lactoglobulin with κ-casein in micelles as assessed by chymosin hydrolysis: effect of temperature, heating time, b-lactoglobulin concentration and pH. J. Agric. Food Chem. 38: 50.
37. Roefs, S.P.F.M. and C.G. de Kruif. 1994. A model for the denaturation and aggregation of β-lactoglobulin. Eur. J. Biochem. 226: 883.
38. Rollema, H.S. Casein Association and micelle formation, in Advanced Dairy Chemistry, volume 1. Proteins, edited by P.F. Fox, Elsevier Science Publishers, England. 111.
39. Schreiber, R. 2001. Heat-induced modifications in casein dispersions affecting their rennetability. Int Dairy J. 11: 553.
40. Singh, H., S.I. Shalabi., P.F. Fox, A. Flynn and A. Barry. 1988. Rennet coagulation of heated milk: influence of pH adjustment before or after heating. J. Dairy Res. 55: 205
41. Singh, H.S. 1993. Heat-induced interactions of proteins in milk. Protein and fat globule modifications-IDF seminar, special issue 9303. 191.
42. Singh, H. and A. Wauguna. 2001. Influence of heat treatment of milk on cheesemaking properties. Int. Dairy J. 11: 543.
43. Smits, P. and J.H. van Brouwershaven. 1980. Heat induced association of β-lactoglobulin and casein micelles. J. Dairy Res. 47: 313.

44. Verheul, M. 1998. Aggregation and gelation of whey proteins. Ph.D. Diss, University of Twente, Enschede, Neth.
45. van Vliet, T. and C.J.A.M. Keetels 1995. Effect of preheating of milk on the structure of acidified milk gels. Neth. Milk Dairy J. 49: 27.
46. Walstra, P.and R. Jenness. 1984. Dairy Chemistry and Physics, John Wiley and sons, Inc, USA.
47. Wauguna, A., H. Singh and R.J. Bennett. 1996. Influence of denaturation and aggregation of β-lactoglobulin on rennet coagulation properties of skim milk and ultrafiltered milk. Food Res. Int. 29: 8: 715.
48. Wheelock, J.V. and A. Kirk. 1974. The role of β-lactoglobulin in the primary phase of rennin action on heated casein micelles and heated milk. J. Dairy Res. 41: 367.
49. White, J.C.D. and D.T. Davies. 1958. The relation between the chamical composition of milk and the stability of the caseinate complex. I. General introduction, description of samples, methods and chemical composition of samples, J. Dairy Res. 25: 236.
50. Wilson, G.A. and J.V. Wheelock. 1972. Factors affecting the action of rennin in heated milk. J. Dairy Res. 39: 413.
51. Holt, C., Yearbook Hannah Research, 1994

Chapter 2: Gelation mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase cross-linked casein micelles

Abstract

Casein micelles in milk are colloidal particles consisting of four different caseins and calcium phosphate of which each can be exchanged with the serum phase. The distribution of caseins and calcium between the serum and micellar phase is pH and temperature dependent. Furthermore, upon acidification casein micelles loose their colloidal stability and start to aggregate and gel.

In this paper we studied two ways of acid-induced gelation, i.e. 1] acidification of milk at temperatures of 20 to 50°C and 2] deceasing the pH at 20°C to just above the gelation pH and subsequently inducing gelation by increasing the temperature. These two routes are called T-pH and pH-T, respectively. The gelation kinetics and the final gels obtained are affected by the gelation route applied. The pH-T milks gel at higher pH and lower temperature and the gels formed are stronger and show less susceptibility to syneresis. By using intra-micellar cross-linked casein micelles, where release of serum caseins is prevented, we demonstrated that in unheated milk serum caseins play a key role in gelation kinetics and characteristics of the final gels formed. This mechanism is presented in a model and is relevant for optimising and controlling industrial processes in the dairy industry, such as pasteurisation of acidified milk products.

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Introduction

Casein micelles are colloidal particles consisting of α_{s1}-, α_{s2}-, κ- and β-casein and calcium phosphate. The micelles are stable at the natural pH of milk, i.e. 6.7, due to the presence of a hairy brush of κ-casein at the surface of the micelle. The casein micelles tend to aggregate if the brush stability is changed, e.g. by decreasing the pH, renneting or addition of alcohol (5, 6, 12, 15), which ultimately leads to gel formation. The iso-electric point of casein micelles is at approximately pH 4.6. Approaching this point by gradually lowering the pH means that the solvency of the brush decreases. The stability of the brush remains intact to about pH 5 (at 20°C). Then, over a small range of pH decrease the brush collapses and the casein micelles aggregate and form a gel (8, 14).

Gel formation depends on the temperature of acidification. Acidification at low temperatures decreases the pH at which a gel is formed (gelation pH) considerably compared to conditions at room temperature (7). Results obtained with sodium caseinate demonstrated that increasing the acidification temperature from 20 to 40°C increases the gelation pH and thus decreases the gelation time at a fixed pH (10). The permeability of the sodium caseinate gels is increased and as observed by confocal scanning laser microscopy coarser gels are formed (11). As demonstrated by Arshad et al. (1) the storage modulus decreases with increasing acidification temperature. These effects are attributed to altered properties of the casein micelles at higher temperatures due to stronger hydrophobic interactions: lower voluminosity, less deformability and hardly any serum casein release (2, 3). At lower temperatures fewer hydrophobic interactions are present, which would allow particles to aggregate with a larger number of bonds between two particles and serum caseins, thereby causing fewer rearrangements during gel formation. The low G' values for gels formed at higher temperatures may be due to extensive rearrangements during gel formation as fewer bonds between the particles are formed. This results in the formation of dense clusters of aggregated particles which in turn aggregate to form a gel. From these dense clusters many particles would hardly contribute to the rigidity of the network, resulting in a weak gel (10).

An alternative way to induce gel formation is warming up of cold-acidified milk. At very low temperatures (4°C) no gelation takes

place at the iso-electric point (4.6) of casein micelles (12), due to strong reduction of hydrophobic interactions. Increasing the temperature of acidified milk (pH 4.6) to 30°C causes gelation around 10°C (7, 17). Variations in the pH of milk also change the temperature at which acidification takes place: the higher the pH the higher the temperature required for gelation (7). In a range of 20 to 50°C the temperature at which the gels are aged hardly affects the G', but only if a slow temperature increase from 4°C to the ageing temperature is applied (0.5°C/min). An instantaneous temperature increase hardly affects the G' at 30°C, but causes a severely decreased G' at 50°C. Also the permeability increases significantly with higher rates of heating to the ageing temperature (12). Aggregation of serum caseins during temperature increase are believed to play an important role in this temperature-induced gelation of cold-acidified milk.

In this chapter two ways of inducing gel formation in unheated milk are discussed, i.e. warming up of cold acidified milk or acidifying directly at higher temperatures. In both cases pH and temperature determine the rheology and microstructure of the final gels. Very little research has been performed in which these two ways of inducing gel formation have been compared (2, 17), although very large effects were observed in gel strength. Gels formed by acidification in the cold (4°C) followed by a temperature increase to 30°C show a 20 times higher G' (17, 18) and a lower permeability compared to milk acidified to the same pH at 30°C. These effects were attributed to the way the micelles are linked together, namely by straight or by bent strands due to temperature-dependent changes in the voluminosity of the casein micelles (2, 17). pH- and temperature-dependent processes occurring in milk, like solubilisation of calcium phosphate (4, 9, 13) and release of serum caseins (3, 9), were not taken into account.

Obtaining a 20-fold increase of G' with the same starting material, but with an alternative way of gel formation, might be very relevant for dairy-derived products. However, the current knowledge (2, 17) is based on gels formed by warming up of cold-acidified milk (4°C). In practice, acidification by microorganisms will not take place at 4°C, but at temperatures of 20°C and higher. Therefore in this paper we investigated milk gels formed with milk which was either acidified by GDL at 20°C and then warmed up (20-50°C), or first warmed up (20-50°C) and then acidified with GDL. The acidified

milks prior to warming up were in all cases liquid, as the pH region studied started just above the gelation pH of milk at 20°C and up to higher values (pH 5.0-5.5). Clear differences between the two routes of gel formation where observed in the gelation mechanism and the characteristics of the final gels. The mechanism inducing these differences, in which serum caseins appear to play a crucial role, is discussed in this chapter.

Material and Methods

Reagents and chemicals

Glucono-δ-lactone (GDL) and D-gluconic acid (sodium salt) were purchased from Sigma Chemicals (St.Louis, MO.USA). Transglutaminase was obtained from Ajinomoto Co. Inc. (Japan); sodium azide was purchased from BDH Laboratories Supplies (Poole, England).

Skimmed milk and whey-protein-free milk

Low-heat skim milk was prepared by dissolving 10.45 g of milk powder (Nilac; NIZO food research Ede) in 100 g distilled water while gently stirring. Whey-protein-free (WPF) milk was prepared by dissolving 8.95 g of WPF milk powder (microfiltration / ultrafiltration; NIZO food research, Ede) in 91.05 g distilled water (8.95%, w/w). After stirring for 1 hour at 45°C, 0.02% (w/w) sodium azide was added to prevent bacterial growth and the milks were kept overnight at 4°C before use. The initial pH of the milks was 6.67 (± 0.01). Prior to experiments skim milk and WPF-milk (stored at 4°C) were stirred for 2 hours at 20°C before further usage.

The reconstituted skim milk consisted of 2.60% (w/w) casein, 0.50% (w/w) whey protein, 4.84% (w/w) lactose, next to non-protein nitrogen, salts and organic acids. These values are similar to average values known for raw skim milk (19). Reconstituted WPF-milk was prepared slightly more concentrated than finally required. This allowed dilution with transglutaminase (see next paragraph) to a final concentration of 8.4%. The concentration of WPF-milk was standardized on the casein concentration in reconstituted skim milk. The WPF-milk (8.4%) consisted of 2.64% (w/w) casein, 0.02% (w/w)

whey proteins, 4.44% (w/w) lactose, next to non-protein nitrogen, salts and organic acids.

Preparation of intra-micellar cross-linked micelles

The required amount of WPF milk was equilibrated for 1 hour at 40°C. A 2% (w/w) transglutaminase solution (activity 20 units/g) was prepared by dissolving the enzyme powder in distilled water, stirring for 2 hours at room temperature and subsequent filtration (5μm). The clear, brownish enzyme solution was used to reach a final activity in the milk of 50U/g protein (protein content of WPF milk is 2.8%), and this was incubated for 1 hour at 40°C. The solution was transferred into glass tubes (5 ml per tube) and heated for 25 min at 90°C in order to inactivate the enzyme. After cooling under tap water to room temperature the milk was either used directly for further experiments or stored overnight at 4°C. This milk will be referred to as CL WPF milk. WPF-milk non-cross-linked (non-CL WPF milk) was treated in the same way as described above but instead of transglutaminase double-distilled water was added. Before further use the milks stored at 4°C were stirred for 2 hours at room temperature. The inactivation of the transglutaminase was checked by performing light scattering experiments on acid-induced gels dissolved in SDS. A heat treatment of 25 minutes was sufficient to prevent any cross-linking during acidification. At shorter times a clear increase in size was observed.

T-pH route and pH-T route

The different milks were subjected to gelation according to two different routes, i.e. pH-T and T-pH, as shown schematically in Figure 2.1. In the T-pH route, milk was first warmed up to the required acidification temperature, i.e. 20, 32, 43 or 50°C, and kept for 75 min at this temperature. Subsequently it was acidified with GDL. At each temperature different amounts of GDL were used: 1.2% (w/w) at 20 and 32°C, 1% at 43°C and 0.8% at 50°C. GDL hydrolyses into gluconate and a proton in a 1:1 molar ratio. In all samples the amount of gluconate ions was adjusted, by addition of sodium gluconate, to the amount formed in 1.2% GDL. The pH at which gelation occurred was determined by diffusing wave spectroscopy (DWS).

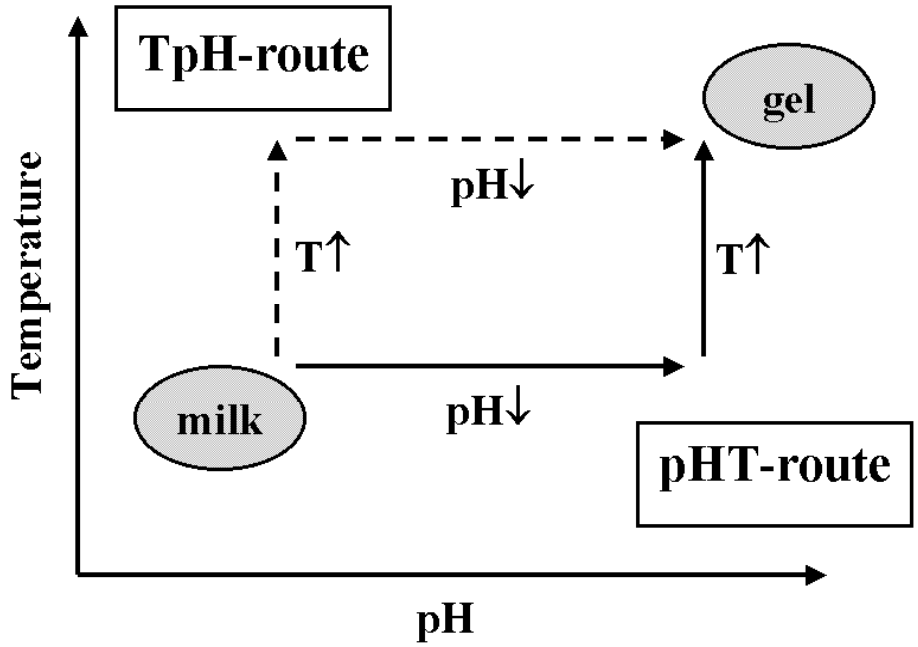

Figure 2.1: Schematic representation of the pH-T and T-pH route, the dashed line indicates the T-pH route, the non-dashed line the pH-T route.

In the pH-T route different amounts of GDL were added to obtain different pH values after 66h of acidification at 20°C. In all cases the final pH values reached were above the gelation pH. For skimmed milk, a range of 0.70 to 0.92% GDL (w/w) was applied, for non-CL WPF-milk 0.70 to 0.95% (w/w) and for CL WPF-milk 0.65 to 0.90% (w/w). The amount of gluconate ions in the samples was adjusted, by addition of sodium gluconate, to the amount formed after hydrolysation of 1.2% GDL. After adding GDL and sodium gluconate, samples were gently stirred for about 1 min and acidified for 66 hours. Subsequently the acidified milks were warmed up at a rate of 0.2°C/min, from 20°C to 50°C. The temperature at which gelation occurred was determined by DWS.

Determination of the gelation point by DWS

Light from a 5 mW He-Ne laser (632.8 nm) was passed through a multi-mode fibre into the milk. The back-scattered light was monitored by a single-mode fibre located at 3 mm from the input fibre. The scattered light was detected with a photo multiplier tube (ALV SO-SIPD), transforming the light signal into an electronic signal, which was fed to a PC-interfaced autocorrelator board (Flex 5000; Correlator.com) and resulted in an autocorrelation function. The time at which the autocorrelation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{½}$ (15). The gelation pH and

gelation temperature are defined as the point where the $\tau_{½}$ (pH) and $\tau_{½}$ (T) curve significantly diverge from the baseline. Prior to acidification or warming up the $\tau_{½}$ value of the starting sample is measured by averaging 5 measurements. All $\tau_{½}$ values obtained during monitoring were normalised with this starting value, resulting in $\tau_{½}$ (pH) and $\tau_{½}$ (T) curves starting at a value of $\tau_{½}$-normalised of 1.

Photographs of pH-T and T-pH milk gels

Skimmed milk was warmed up to 20, 32, 43 and 50°C and acidified with 0.88% GDL, which resulted in the T-pH samples. For the pH-T samples skimmed milk was acidified with 0.88% GDL at 20°C, and subsequently warmed up to 32, 43 and 50°C. The rate of warming up, the times of acidification, and the addition of sodium gluconate were the same as described above. The 43 and 50°C T-pH samples were acidified for 5 hours; the 32°C sample was incubated overnight. In all cases 5 ml of milk was put in 8 ml tubes. After the two routes were performed the samples were cooled down to 20°C, turned upside down and photographs were taken. In case of non-CL WPF milk and CL WPF-milk respectively 0.95% and 0.88% GDL was added.

Dynamic light scattering experiments

Dynamic light scattering experiments were done as outlined by Verheul et al. (16), using a Malvern Autosizer IIC Submicron Particle Size Distribution Analyzer. The system consisted of a Malvern PCS41 optics unit with a 5 mW He-Ne laser, and a Malvern K7032-ES correlator used in serial configuration. The Autosizer IIC worked at a fixed scattering angle of 90^{o} and the wavelength of the laser beam was 632.8 nm. The sample was diluted 500 times with simulated milk ultrafiltrate. The quartz cuvette (10 mm) containing the sample was thermostatted by a Joule-Peltier thermostat (20 ^{o}C). The apparent diameter of the protein particles in solution was calculated from a cumulant fit of the intensity autocorrelation function. Before analysis, samples were filtered through a low-protein-binding membrane (5 μm; Millex-SV, Millipore Corporation, Bedford, MA., USA).

pH-T: rate of temperature increase

The acidified skimmed milk samples were prepared according to the pH-T route. The samples were subjected to three different rates of temperature increase; i.e. instantaneously, 0.2°C/min and 0.02°C/min. The gelation points of 0.2 and 0.02°C/min were determined by DWS. The instantaneous temperature increase was performed by putting tubes for 20 min at one temperature and determining the gelation visually. The slightest start of flocculation was judged as the gelation point.

Serum casein determination in CL and non-CL WPF milk

Non-CL and CL WPF milk were subjected to ultracentrifugation at pH 6.7 (milk pH) and 5.3 (0.7% GDL) at 30000g for 60 min. The samples were analysed by capillary electrophoresis (Beckman P/Ace 5000; Beckman Coulter Inc.) with a capillary (Agilent, μSil-wax, internal diameter 0.05 mm) of 60 cm length. The samples were injected for 20 seconds with a pressure of 0.5 psi. The electrophoresis was carried out at 45°C and a voltage of 25 kV towards the cathode and detection was at 214 nm. The electrophoresis was carried out in 6M urea and under reducing conditions by addition of DTT.

Results and Discussion

Intra-micellar cross-linking of WPF-milk

Table 2.1 shows the effect of cross-linking of WPF-milk on the size of the casein micelles and the amount of serum casein present in the supernatant obtained by ultra centrifugation at pH 6.7 and 5.3. The table demonstrates that the size of the casein micelles was changed only slightly by the transglutaminase treatment applied. A slight reduction is size was observed. In case of non-CL WPF-milk around 20% of casein was released in serum at pH 6.7 and 50% at pH 5.3. Hardly any serum casein could be detected in CL-WPF milk.

The size measurements performed, showing a slight decreases in size, exclude inter-micellar cross-linking as this would have caused an increase in size. The decreased micellar size is attributed to the temperature at which cross-linking took place, i.e. 40°C. At this

Milk	Diameter (nm) of micelles	% casein in serum phase at pH 6.7	% casein in serum phase pH 5.3
Non-CL WPF	233 ± 2	21.8	51.1
CL WPF	207 ± 3	0.6	1.7

Table 2.1: Effect of cross-linking (CL) of WPF-milk by transglutaminase on micellar size and on serum casein level in milk at pH 6.7 and 5.3 at 4°C

temperatures the size of casein micelles decreases compared to the size as room temperature. The cross-linking process fixes the state of the casein micelle, making the size no longer dependent on the temperature. The amount of serum casein present in supernatants of WPF-milk is in agreement with that reported by Dalgleish (3). Cross-linking of the milk with transglutaminase almost completely prevents release of caseins in the serum, indicating that the micelles are intra-micellar cross-linked. These intra-micellar cross-linked casein micelles hardly release serum casein upon decreasing temperature and pH.

CL and non-CL WPF milk were subjected to acid-induced gel formation via the T-pH and pH-T route (schematically represented in Figure 2.1). The gelation points were determined with DWS by plotting $\tau_{½}$ as function of temperature or pH. The pH and temperature at which gelation occurred is plotted in the figure. In all cases the milk samples at 20°C remained liquid, as conditions were chosen in such a way that the gelation point was not reached. In Figure 2.2 the gelation points and photographs of the final gels obtained at 32, 43 and 50°C of non-CL WPF milk are depicted. Gel formation via the pH-T route resulted in firm gels without serum release at 32, 43 and 50°C. A stable T-pH gel was formed at 32°C, while at 43 and 50°C the gels were unstable and serum was released. The pH-T gelation points were situated below the T-pH gelation points with a clear gap in between.

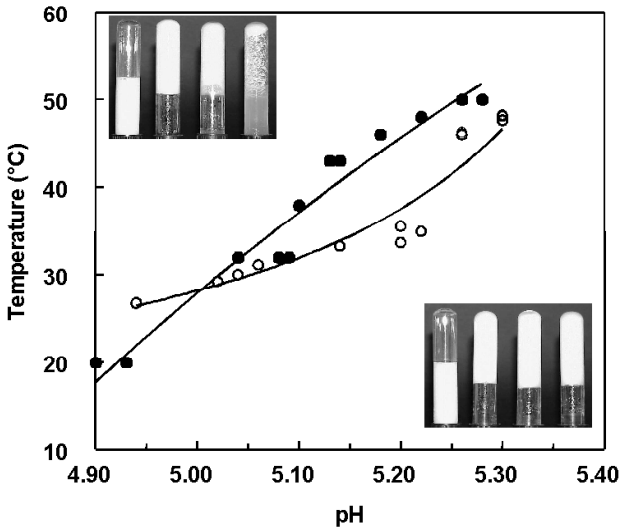

Figure 2.2: The gelation points of T-pH (●) and pH-T (○) of non-CL WPF milk represented by the pH and temperature at which gelation started. The photographs taken at room temperature, show the corresponding gels formed in tubes which were turned upside down after the gelation process was finished. The photographs representing, from left to right, milk at 20, 32, 43 and 50°C, are situated in the top left corner (T-pH route) and in the bottom right corner (pH-T route). The lines are drawn to guide the eye.

The above results demonstrate clearly that the gelation points and the properties of the final gels are very dependent on the way the gels are prepared. Apparently, the gelation is not just determined by final pH and temperature, but also by the sequence they are applied. Preparation of gels by the pH-T route results in stronger gels with less serum separation. Although the pH (4.6) and temperature (4 to 30°C) condition applied are rather different, this is comparable with the results of Bremer (2) and van Vliet et al (17). In these papers the reason for obtaining stronger gels via the pH-T route was attributed to the way the casein micelles are linked, namely via straight (pH-T) or bent strands (T-pH). The straightening supposedly stemmed from the differences in voluminosity of casein micelles. Around 10°C a gel is formed, but the temperature is further increased to 30°C causing a shrinkage of the micelles by 15%, which results in straightening of the strands. Although this might explain the effects on gel strength as observed in Figure 2.2, it cannot explain the differences in gelation point. Therefore, another factor appears to determine the gelation properties. There are two processes in milk which are either pH or temperature dependent, i.e. solubilisation of calcium phosphate (4, 9, 13) and release of serum caseins (3, 9). As shown in table 2.1, cross-linking of casein micelles is a very effective way to prevent release of

serum caseins, while it is not likely to hamper solubilisation of calcium phosphate due to the open structure of the micelle.

Gel formation via T-pH and pH-T with CL WPF milk results in firm gels without any serum release at all temperatures and for both routes applied (Figure 2.3) . Gelation points of both routes are very similar, although that for the pH-T route is situated slightly higher. These results clearly differ from those of non-CL WPF-milk, where the T-pH gelation points were situated above the pH-T points with a clear gap in between and the gels of the T-pH route were unstable at 43 and 50°C. Therefore, we conclude that cross-linking of the casein micelles results in a gelation behaviour which is almost independent of the way gelation is induced. This indicates the relevance of serum caseins in the gelation process. Solubilisation of calcium phosphate seems not to be a very relevant factor in determining the differences between the gelation processes. This is in agreement with Dalgleish and Law (4), who observed a pH-dependent calcium solubilisation, but hardly any effect of temperature in the range of 4 to 30°C. Additionally, experiments where the serum calcium content was changed by addition of calcium chloride or withdrawal of calcium by EDTA demonstrated hardly any effect of calcium on the gelation points (results not shown).

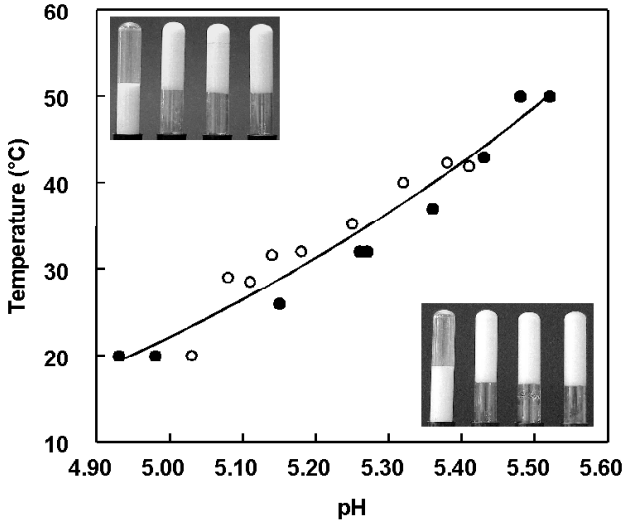

Figure 2.3: The gelation points of T-pH (●) and pH-T (○) of CL WPF milk represented by the pH and temperature at which gelation started. The photographs show the corresponding gels formed in tubes which were turned upside down after the gelation process was finished. The photographs representing from left to right, milk at 20, 32, 43 and 50°C, are situated in the top left corner (T-pH route) and in the bottom right corner (pH-T route). The line is drawn to guide the eye.

The gelation points of the T-pH route of CL WPF-milk are lower than for non-CL WPF-milk. Apparently, cross-linking of the hairy brush destabilises the casein micelle probably due to reduced flexibility of the brush. Therefore, it is not possible to compare the gelation points between CL and non-CL WPF-milk directly, which could have provided additional information about the effect of serum caseins on the gelation point.

Model

About 10% serum caseins are present at pH 6.7 and 20°C. Increasing the temperature decreases this amount to almost 0% (T-pH), while decreasing the pH to just above the gelation point results in release of 30% serum caseins (pH-T) (3). This causes a milk system prior to gelation with 30% difference in serum casein present in the supernatant. Decreasing the pH at higher temperatures (T-pH) causes collapse of the hairy brush and subsequent aggregation of the micelles, and finally gel formation. At these temperatures hardly any serum casein is released during acidification and it will therefore not interfere with gelation. To the contrary at 20°C and a pH of 5.1 a considerable amount of serum casein is present. During temperature increase (pH-T) the solvent quality decreases and the 30% serum casein will associate or re-associate with the casein micelle. Due to

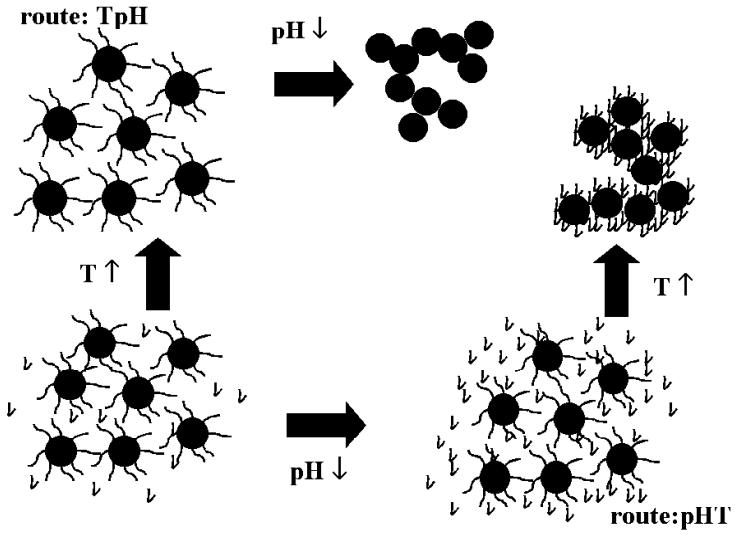

Figure 2.4: Model depicting the contribution of serum caseins to the induced gelation via the pH-T and T-pH routes. The large filled circles represent casein micelles. The hairs protruding from the micelles represent the κ-caseins present on the surface of the micelle. The small v-shaped symbols represent the serum caseins.

the decreased electrostatic repulsion this re-association is probably more an association with the κ-caseins on the surface of the micelle. In this system the associated serum casein molecules will contribute to the gel formation and may act as bridging material. Additionally, they will affect the pH of gelation, as the calcium-sensitive caseins are not protected by a hairy brush of κ-casein as in the T-pH route. Therefore in the pH-T route the increasing concentrations of calcium due to pH reduction will cause precipitation at higher pH values. Changing the gelation process from T-pH to pH-T will lead to higher gelation pH and stronger gels that show less rearrangements and syneresis. Figure 2.4 presents a schematic representation of this model.

Cross-linking of the casein micelles will prevent the formation of serum caseins and therefore the gelation points and final gels obtained via pH-T and T-pH will be very similar. It was also observed that the gels consisting of CL WPF-milk formed via the T-pH route are more stable than the gels consisting of non-CL WPF-milk. It is believed that this is due to a more effective use of the casein fraction in milk. The crosslinked κ-casein brush is no longer able to collapse, therefore the crosslinked micelles occupy a higher volume fraction at low pH than non-crosslinked miceles. Additionally, the κ-caseins of the micelles will interfere which will result in more stable gels.

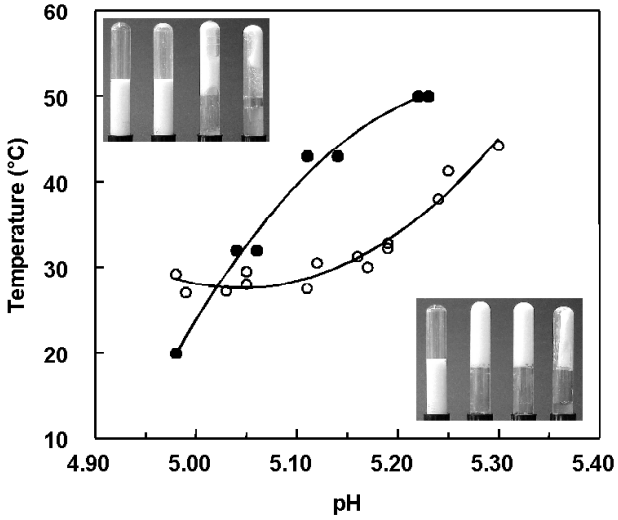

Figure 2.5: The gelation points of T-pH (●) and pH-T (○) of skimmed milk represented by the pH and temperature at which gelation started. The photographs show the corresponding gels formed in tubes which were turned upside down after the gelation process was finished. The photographs representing from left to right, milk at 20, 32, 43 and 50°C, are situated in the top left corner (T-pH route) and in the bottom right corner (pH-T route). The lines are drawn to guide the eye.

Acid-induced gelation of skimmed milk via the T-pH and pH-T route

The same experiments as performed for CL and non-CL WPF milk were carried out with skimmed milk. WPF milk was used as a model system and due to the absence of whey proteins and the blank treatment applied to the non-CL WPF samples it is not necessarily representative of skimmed milk which is used in the preparation of acid-milk products. Figure 2.5 depicts the T-pH and pH-T gels and gelation points of skimmed milk. The gelation points are very similar to those of non-CL WPF milk. There are some differences in the gels formed: the pH-T milk gel formed by warming up to 50°C is not stable and the T-pH gel formed at 32°C remains liquid. However, apart from these two gels non-CL WPF milk and skimmed milk behaved very similarly, i.e. a clear gap separated the gelation points of the T-pH and pH-T routes and clear differences were observed in the final gels obtained at the different temperatures. This demonstrates that the treatment applied to obtain non-CL WPF-milk and the presence of native whey proteins in skim milk do not cause major changes in the gelation mechanism. Therefore, we conclude that the model as presented in Figure 2.3 is also valid for skimmed milk.

pH-T route: rate of temperature increase

The effect of the rate of temperature increase on gel formation of skimmed milk according to the pH-T route is presented in Figure 2.6. The temperature increase was varied between 0.02°C/min and an instantaneous increase, but no effect was observed on the gelation points of the pH-T route, indicating that the rate of temperature increase does not affect the gel formation. It is generally known that re-association of serum casein with the micelle is a slow process with a time scale of one hour or more. At the lowest heating rate there is ample time for re-association. However, no effect of the rate on the gelation points was observed, indicating that it is not a time-dependent process within a time scale of 24 hours. Apparently, the way casein micelles are present during the T-pH route is not restored during very slow warming up of milk acidified by the pH-T route. The association and re-association of serum casein during temperature increase is determined by the balance of electrostatic attractions and hydrophobic interactions and appears to result in a rather stable solution.

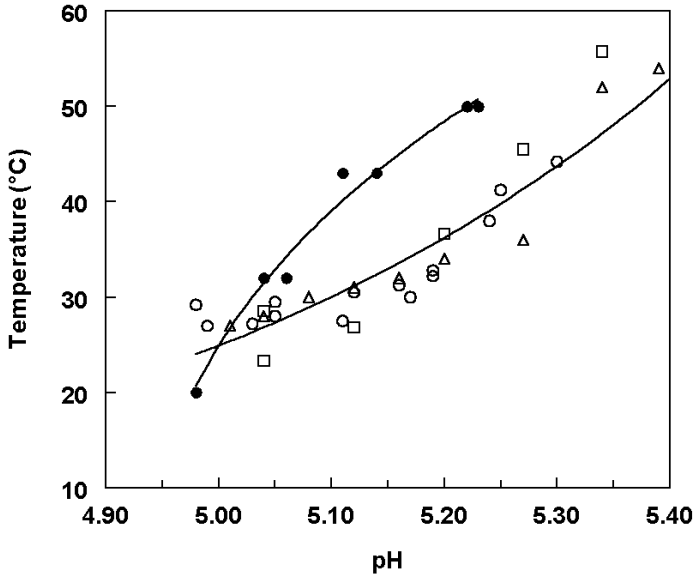

Figure 2.6: Gelation points of skimmed milk represented by the pH and temperature at which the gelation started: gelation induced by the T-pH route (●), gelation induced by the pH-T route at a temperature rate of 0.02°C/ min (□), 0.2°C/min (○) and instantaneously (△). The lines are drawn to guide the eye.

Conclusions

Summarising, we demonstrated that pH-T gels start to gel at higher pH values and result in more stable gels than T-pH gels. Serum caseins appear to play a crucial role in determining the differences in T-pH and pH-T gel formation, which was presented in a model. The rate of warming up did not affect the start of gel formation, indicating that the process is not very time dependent, but mainly driven by electrostatic interactions. Understanding this mechanism should be of help in optimising and controlling industrial processes in the dairy industry, like pasteurisation of acidified milk products.

References

1. Arshad, M., M. Paulsson, and P. Djemek. 1993. Rheology of buildup, breakdown, and rebodying of acid casein gels. J. Dairy Sci. 76: 3310.
2. Bremer, L.G.B., B.H. Bijsterbosch, R. Schrijvers, T. van Vliet and P. Walstra. 1990. On the fractal nature of the structure of acid casein gels. Colloids and Surfaces. 51: 159.
3. Dalgleish, D.G. and A.J.R. Law. 1988. pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. J. Dairy Res. 55: 529.

4. Dalgleish, D.G. and A.J.R. Law. 1989. pH-induced dissociation of bovine casein micelles II. Mineral solubilization and its relation to casein release. J. Dairy Res. 56: 727.
5. Horne, D.S. and C.M. Davidson. 1986. The effect of environmental conditions on the steric stabilization of casein micelles. Colloid. Pol. Sci. 264: 727.
6. Horne, D.S. and J. Leaver. 1995. Milk proteins on surfaces. Food Hydrocolloids. 9. 2: 91.
7. de Kruif, C.G. and S.P.F.M. Roefs. 1996. Skim milk acidification at low temperatures: A model for the stability of casein micelles. Neth. Milk Dairy J. 50: 113.
8. de Kruif, C.G. and E.B. Zhulina. 1996. κ-Casein as a polyelectrolyte brush on the surface of casein micelles. Colloids and Surfaces A. 117: 151.
9. Law, A.J.R. 1996. Effect of heat treatment and acidification on the dissociation of bovine casein micelles. J. Dairy Res.63: 35.
10. Lucey, J.A., T. van Vliet, K. Grolle, T. Geurts and P. Walstra. 1997. Properties of acid casein gels made by acidification with GDL 1: rheological properties. Int. Dairy J. 7: 381.
11. Lucey, J.A., T. van Vliet, K. Grolle, T. Geurts, P. Walstra. 1997. Properties of acid casein gels made by acidification with GDL 2: syneresis, permeability and microstructural properties. Int. Dairy J. 7: 389
12. Roefs, S.P.F.M. 1986. Structure of acid casein gels, Ph.D Diss., Wageningen Agric. Univ., Wageningen, Neth.
13. Singh, H., M.S. Roberts, P.A. Munro and C.T. Teo. 1996. Acid-induced dissociation of casein micelles in milk: effect of heat treatment. J. Dairy Sci. 79: 1340.
14. Tuinier, R. and C.G. de Kruif. 2002. Stability of casein micelles in milk. Accepted for publication in J. Chem. Physics.
15. Vasbinder, A.J., P.J.J.M. van Mil, A. Bot and C.G. de Kruif. 2001. Acid-induced gelation of heat treated milk studied by Diffusing Wave Spectroscopy. Colloids and Surfaces B. 21: 245. Chapter 6 of this thesis.
16. Verheul, M., S.P.F.M. Roefs and C.G. de Kruif. 1998. Kinetics of heat-induced aggregation of β-lactoglobulin. J. Agric. Food Chem. 46: 896.
17. van Vliet, T. and C.J.A.M. Keetels. 1995. Effect of preheating of milk on the structure of acidified milk gels. Neth. Milk Dairy J. 49: 27.
18. van Vliet, T., S.P.F.M. Roefs, P. Zoon and P. Walstra. 1989. Rheological properties of casein gels. J. of Dairy Res. 56: 529
19. Walstra, P. and R. Jenness. 1984. Dairy Chemistry and Physics, John Wiley and sons, Inc, USA.

Chapter 3: Heat-induced casein-whey protein interactions in milk

Abstract

Heating of milk is an essential step in the processing of various dairy products, like for example yoghurt. A major consequence of the heat treatment is the denaturation of whey proteins which either associate with the casein micelle or form soluble whey protein aggregates. This paper describes a quantitative study on the distribution of denatured whey proteins over whey protein aggregates and casein micelles. The distribution is thought to be related to changed acid-induced gelation properties and textural properties of milk derived products. Quantitative description will allow better control and tuning of the final gel properties.

By combination of enzymatic fractionation and capillary electrophoresis we were able to quantitatively determine the distribution of denatured whey proteins after heat treatment. More severe heat treatment at the natural pH of milk caused more denaturation, but the ratio of denatured whey proteins associated with the casein micelle and present in aggregates remained constant. We clearly demonstrated that the observed shift in gelation pH of heated milk is linearly correlated with the two fractions of denatured whey proteins.

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Introduction

The main reason for heat treatment of milk is to improve its keeping quality by decreasing the number of living microorganisms and to achieve desirable properties in the final product (33). Upon heat treatment of milk above 60°C several processes take place, of which denaturation of whey proteins is the most obvious (7, 26, 27, 34). The most abundant whey protein is β-lactoglobulin (β-lg) in which a heat-induced conformational change exposes a reactive thiol group. This thiol group can form disulfide bonds with other cysteine-containing proteins, like β-lg or bovine serum albumin, or with proteins having disulfide bridges, like α-lactalbumin (α-lac), κ- and α_{s2}-casein. The latter process occurs through thiol group-disulfide bridge exchange reactions, resembling a polymerisation process where heat-denatured β-lg is the initiator (12, 16, 17, 30, 39). Interaction of β-lg with κ-casein, present at the exterior of the casein micelle like a hairy brush, leads to coating of the casein micelles with β-lg. Interactions of β-lg with cysteine-containing serum caseins might lead to casein-whey protein aggregates. Additionally, interactions of β-lg with cysteine-containing whey proteins, like α-lac and β-lg molecules, result in the formation of whey protein aggregates. To summarise, heat treatment of milk results in a complex mixture of native whey proteins and denatured whey proteins present as whey protein aggregates, casein-whey protein aggregates and whey protein coated casein micelles.

Heat treatment of milk has a profound effect on the acid-induced gelation properties. It causes a shift in gelation pH towards higher pH values (11, 15, 23, 39). This is attributed to the interaction of β-lg with the casein micelle. β-lg tends to neutrality at a higher pH than casein micelles (5.2 instead of 4.6) (39). Gels formed of heated milk have an increased gel hardness and higher storage modulus (G') (23, 24, 28, 40, 42) and shows less susceptibility to syneresis (8, 9) compared to gels made of unheated milk. The increased gel hardness is partially caused by the changed surface properties of the whey-protein-coated casein micelles and partially by the disulfide interactions occurring during the gel state (40). It is generally assumed that the altered properties of heated milk are related to the total degree of whey protein denaturation. It is known that the composition of the complex mixture of denatured whey proteins and

casein micelles is dependent on the pH of heat treatment, temperature, total protein concentration, the ratio between casein micelles and whey proteins, the salt concentration and so on (2, 3, 6, 7, 10, 20, 21, 22, 25, 27, 35). However, limited research has been performed which relates the changed gelation properties of heated milk to the partition of denatured whey proteins between aggregates and micellar coating.

In order to study this relation, fractionation of the denatured whey protein is required as this will enable quantitative measurements of the distribution of whey proteins in aggregates and as coating. In most papers ultracentrifugation is used as a fractionation technique, quite often in combination with density measurements of SDS-PAGE gels (6, 10, 34, 27). By using ultracentrifugation as a fractionation tool very large g-forces are applied on the sample, causing a risk of precipitating soluble whey protein aggregates. Noh and Richardson (25) proposed an alternative fractionation method based on renneting, which has the major advantage that low g-forces are required. In this study we used this fractionation method in the absence of calcium to prevent the risk of calcium-induced precipitation of whey protein aggregates (5, 14). We combined this fractionation method with a more accurate analysis technique than the currently used methods, namely capillary electrophoresis (CE). SDS-agarose gel electrophoresis (1) was used to characterise the aggregates in the supernatant with respect to size and to study the interactions occurring between casein micelles and whey proteins. The results of this quantitative study, performed with a unique combination of a mild fractionation method using enzymes and quantification by CE, are related to the changed gelation characteristics of milk heated at temperatures ranging from 70 to 90°C.

Materials and Methods

Skim milk

Skim milk heated for 10 seconds at 72°C was obtained from the pilot plant at NIZO food research, Ede. Sodium azide (0.02% w/w) was added to prevent bacterial growth and the milk was stored at 4°C.

Whey Protein Isolate solution

Whey Protein Isolate (Bipro; Davisco Food International Inc., USA) solution was prepared at a concentration of 9% (w/w). As determined by Tuinier (38) the isolate powder consisted of β-lg (71%), α-lac (12%), bovine serum albumin (5%) and immunoglobulin (5%). The total amount of proteins in the powder is 93% of which 92% is native, i.e. soluble at pH 4.6. It further contains lactose (0.3%), ash (1.8%) and water (5%). The whey protein solution was stirred for 2 hours at room temperature and subsequently filtered though a 0.45μm filter; 0.02% (w/w) NaN_3 was then added. Heating this solution for 2 hours at 68.5°C led to the formation of aggregates with a hydrodynamic diameter of 62 nm which was determined by dynamic light scattering (1).

Heat treatment of milk

Glass tubes (volume 8 ml, diameter 1 cm) were filled with 5 ml of milk, closed with a screw cap and heated for 10 min in a water-bath at 80°C and cooled with tap water to room temperature.

Fractionation of whey proteins

Acetic acid precipitation

An amount of 0.4 g of milk was mixed with 0.8 g of distilled water (40°C) and 40 μl acetic acid (10%) in an Eppendorf tube (2 ml). After mixing (vortex) and 10 min waiting, 40 μl of sodium acetate (1M) and 0.72 g of distilled water were added and the solution was mixed again. After 1 hour of incubating at ambient temperature the solution was centrifuged for 5 min at 3000g. The concentration of native protein present in the supernatant was determined by capillary electrophoresis (CE).

Rennet precipitation

Renneting of the milks was performed essentially based on the method of Noh et al. (25), but without addition of calcium chloride. The concentration of whey proteins, i.e. native and aggregated, was determined by CE.

Capillary electrophoresis

The fractionated samples were analysed by CE (Beckman P/Ace 5000; Beckman Coulter Inc.) with a capillary (Agilent, μSil-wax, internal diameter 0.05 mm) of 60 cm length. The samples were injected during 20 seconds with a pressure of 0.5 psi. The electrophoresis was carried out at 45°C using a voltage of 25 kV towards the cathode and detection was at 214 nm. The experiments were performed in buffer containing 6M urea and dithiothreitol (DTT).

Preparation of SDS gel electrophoresis samples

The supernatant obtained after centrifugation of the rennet-treated milk and the milks themselves were diluted 1 to 1 with 20 mM Bis-Tris buffer and 5% SDS (pH 7.0) (1); the precipitate obtained after centrifugation of the rennet-treated milk was solubilized by replacing the supernatant with an equal volume of the Bis-Tris buffer. All samples were held overnight at ambient temperature, while the precipitates dispersed in buffer were constantly stirred. In some experiments a treatment with the disulfide-reducing agent DTT (0.05%) was carried out to reduce the disulfide bonds in solubilized samples.

Agarose gel electrophoresis

SDS-agarose gels were prepared at a concentration of 0.4% agarose according to Alting et al. (1). Briefly the electrophoresis buffer consisted of 100 mM Tris, 50 mM sodium acetate, 2 mM EDTA and 0.1% SDS, and was adjusted to pH 7.9 with concentrated acetic acid. Prior to electrophoresis, 5% of a solution containing 60% glycerol and 0.002% bromophenol blue was added to the samples stirred overnight. Per sample 20μl was applied on the gel; in the case of aggregates the sample volume was 100μl. The gels were run with a constant voltage of 50V for approximately 2 hours and finally stained with Phastgel blue R.

Diffusing Wave Spectroscopy

Milks were incubated for 75 min at 32°C, followed by acid-induced gelation achieved by addition of 1.2% GDL. The clotting of the milk was monitored by DWS. Light from a 5 mW He-Ne laser

(632.8 nm) was passed through a multi-mode fibre into the milk. The back-scattered light was monitored by a single-mode fibre located at 3.0 mm from the input fibre. The scattered light was detected with a Photo Multiplier Tube (ALV SO-SIPD and fed to a PC interfaced autocorrelator (Flex 5000, correlator.com). The time at which the autocorrelation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{½}$ (39). The correlation functions were monitored in time at intervals of 2 min. All data were normalised by the $\tau_{½}$ value (average of five measurements) of the same sample prior to GDL addition. This eliminates variations between the samples and fibres.

Results and Discussion

Denaturation of whey proteins: association with the micelles and formation of aggregates

In order to determine quantitatively the distribution of denatured whey proteins between soluble aggregates, whey proteins associated with the casein micelle and native whey proteins fractionation of heated milk was performed. Figure 3.1 depicts the denaturation of α-lactalbumin (α-lac, Figure 3.1a) and β-lactoglobulin (β-lg, Figure 3.1b) in milk as function of heating temperature. The fractionation was performed by an alternative method based on renneting of the milk, separating casein micelles from soluble aggregates and native whey proteins. Subsequently, native whey proteins were separated from aggregates by acid precipitation. A clear decrease in native whey proteins is observed with increasing temperature of the heat treatment. After heating at 90°C more than 95% of all the β-lg and almost 80% of all the α-lac present in milk is denatured. About 25% of both proteins is present in the supernatant as soluble aggregates, while 65% of all β-lg and 50% of all α-lac is found in the pellet after renneting and was thus associated with the casein micelle.

The observed degree of denaturation of α-lac and β-lg and the fact that α-lac denatures to a lesser extent than β-lg is in agreement with the literature (7, 27, 34). The soluble aggregates present in the rennet-induced supernatant consisted mainly of whey proteins, as only a small fraction of the casein was present in the supernatant: less than 5% of total casein was detected in the supernatant of milk heated at 85°C or at lower temperatures and about 10% was detected

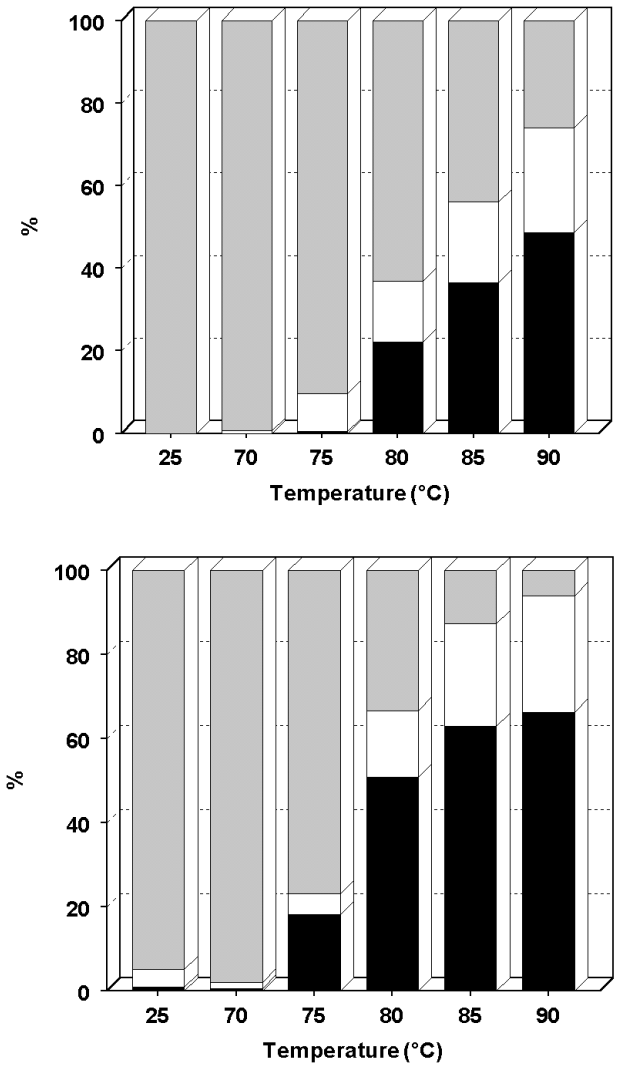

Figure 3.1: Percentage of denaturated α-lac (Figure 3.1a; upper graph) and β-lg (Figure 3.1b; lower graph) present in milk as soluble whey protein aggregates (white bars) and associated with the casein micelle (black bars) after 10 min heat treatment at temperatures ranging from 70-90°C. The grey bars represent native whey proteins.

in milk heated at 90°C, of which κ-casein was almost negligible. These results are in agreement with Singh et al. (34), who observed less than 5% serum κ-casein in unheated milk and less than 3% in milk heated at 90°C. The applied renneting treatment will cause cleavage of κ-casein to insoluble para-κ-casein (43). It is expected that possible non-associated κ-casein-whey protein complexes might precipitate as well, which would explain the very low quantities of κ-casein observed in the serum. Apart from κ-casein, α_{s2}-casein is able to interact with β-lg via thiol group-disulfide bridge exchange reactions; however it is not possible to quantify this casein by CE and therefore no estimations are available of the amount of α_{s2}-casein present in the aggregates. The soluble aggregates will be regarded as

	α-lac	β-lg
Concentration in milk (% w/w)	1.2	3.2
% denatured protein (CE)	80	95
% protein in aggregates (CE)	25	25
% protein associated with the casein micelles (CE)	50	65
Concentration protein in aggregates (%) #	0.24	0.76
Concentration protein in casein micelles (%) #	0.48	1.98
Molar ratio aggregates/casein micelles	0.5	0.38
Molar ratio α-lac/β-lg in aggregates	0.4	
Molar ratio α-lac/β-lg in coating casein micelles	0.31	

concentration protein = concentration in milk * (% denatured protein / 100) * (% protein present in aggregates or casein micelles / 100).

Table 3.1: Determination of the α-lac / β-lg ratio in aggregates and coating of the casein micelles in milk heated at 90°C

whey protein aggregates in the rest of this chapter, although they may contain some κ and α_{s2}-casein.

The percentage of whey proteins associated with the casein micelle after heat treatment at 90°C as observed by us is about 20% lower than observed by Singh (34; 87% of β-lg and 76% of α-lac). Oldfield et al. (27) observed 80% association at pH 6.60 and 60% at pH 6.83, which would indicate that about 70% of the β-lg associates with the casein micelle at pH 6.7. Based on the results of Oldfield et al. (27) the estimated association value for α-lac would be around 50%. These values are in good agreement with the percentages reported in this chapter. Both studies used ultracentrifugation as a fractionation tool, but at different centrifugal force (g) / time combinations. This shows the drawback of ultracentrifugation as fractionation method: soluble aggregates might sediment and be interpreted as part of the pellet, i.e. associated with the casein micelle.

The CE data obtained allow determination of the ratio of α-lac and β-lg in aggregates:coating of the casein micelles and the α-lac/β-lg ratio in aggregates and coating. Table 3.1 shows these ratios for milk heated at 90°C. By comparison of the α-lac/β-lg ratio in aggregates and in the coating of the micelles it is demonstrated that α-lac is more easily incorporated in aggregates than it is involved in the coating of micelles. The α-lac/β-lg ratio is hardly affected by the

temperature of heat treatment in the range 80 to 90°C (not shown in table). The calculated molar ratio of total denatured α-lac and β-lg in the milk heated at 90°C was 2.5, which is identical to the α-lac/β-lg ratio observed in the aggregates. This demonstrates that the aggregates contain a ratio of α-lac/β-lg representative of the whole milk sample, while the whey protein coating of the casein micelles clearly contains more β-lg. These results indicate once more the complexity of whey protein denaturation in heated milk.

Size determination of soluble whey protein aggregates

The soluble aggregates which remained in the supernatant after rennet treatment were characterised for size by SDS gel electrophoresis (Figure 3.2) . Lanes 1 to 6 represent the supernatant of unheated milk and milk heated at temperatures ranging from 70 to 90°C. In lanes 1 to 3 all proteins present in the supernatant show a similar, relative high electrophoretic mobility. In lanes 4 to 6 a protein band is observed with a lower electrophoretic mobility than in unheated milk, representing the aggregates formed during heat treatment. Lane 7 shows a marker of whey protein aggregates with a hydrodynamic diameter of about 62 nm (61.9 ± 1.2 nm).

Whey protein aggregates are observed after heating the milk for 10 min at 80°C and at higher temperatures, which is in agreement with the results shown in Figure 3.1. Also the increased concentration of aggregates at higher temperatures of heat treatment

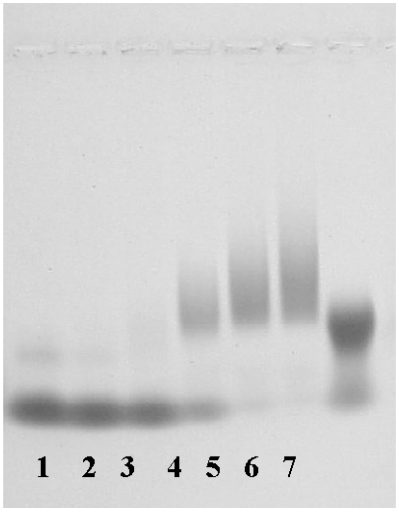

Figure 3.2: SDS agarose gel with in lane 1 to 6 the supernatant of rennet-treated milk, unheated (lane 1), or heated for 10 min at 70, 75, 80, 85 and 90°C (lanes 2 to 6, respectively), and whey protein aggregates of 62 nm prepared from whey protein isolate as a size indicator (lane 7).

corresponds very well with these results. Native whey proteins have a diameter of around 3 nm suggesting an oligomeric structure with a rather broad molecular weight distribution. Previous studies have shown that aggregates with an average size of 280 nm are no longer able to enter the gel (1). Electrophoresis in the presence of SDS provides information on the molecular weight of proteins which is not necessarily the same as size. However, as it is not expected that the aggregates in milk have a completely different shape than aggregates formed in pure whey protein solutions we can use the marker to estimate the size of the aggregates. The aggregates formed in milk during heating behave as if they are slightly larger than 60 nm. At 80°C the molecular weight distribution is smaller than at 90°C; also the concentration seems to increase slightly with higher temperatures, as the intensity of the protein bands stained increased. As far as we know this is the first time that soluble whey protein aggregates formed in milk during heat treatment are visualised and characterised and that the size of these aggregates is estimated.

Whey protein association with micellar κ-casein

To obtain information about the association of whey proteins with the casein micelles, fractionation was performed by renneting the milks causing precipitation of the casein micelles. The pellets obtained after centrifugation of the renneted milk were dissolved in SDS-buffer, inducing disruption of non-covalent interactions. Covalent interactions like disulfide interactions between whey proteins and κ-casein or between the caseins in the casein micelle are not disrupted by SDS.

Unheated milk

Two reference samples were analysed by SDS-agarose gel electrophoresis, i.e. unheated milk and unheated milk with DTT. In both cases the milk was characterised without any fractionation. Unheated milk (lane 2) shows a clear migration pattern of slow- (indicated by arrow [b]) and fast- (indicated by arrow [a]) migrating protein structures (Figure 3.3) . Treatment with DTT (lane 1) changes the slow-migrating band to a faster-moving protein band. Lane 3, containing a sample from the pellet obtained after renneting and centrifugation of unheated milk, demonstrates that the migration velocity of the slow-migrating structure is increased (arrow [c]).

Unheated milk is composed of casein micelles and native whey proteins. Arrow [a] indicates the native whey proteins. Casein micelles in milk are large colloidal particles with a size ranging from 20 to 600 nm (13, 32), mainly linked by non-covalent interactions. After addition of SDS these are disrupted, resulting in monomers (arrow [a]) and disulfide-linked polymers (arrow [b]). These polymers cannot contain casein other than α_{s2}- and κ-casein, because of the lack of cysteine in the other caseins. It has been shown previously that α_{s2}-casein is found both as a monomer and a dimer, whereas κ-casein exists as a disulfide-linked polymer (4, 29, 36, 37) with sizes larger than 500 kDa. The accurate molecular weight could not be determined as the experiments were performed by SDS-PAGE, which has a detection limit of 500 kDa. Our results demonstrate that the casein polymers behave as if they are slightly larger than aggregates with a hydrodynamic diameter of 60 nm, which suggest a relatively high molecular weight. Lane 1 shows that in the presence of DTT the casein polymers are disrupted into monomers; this demonstrates that the caseins are disulfide linked, which is in agreement with the

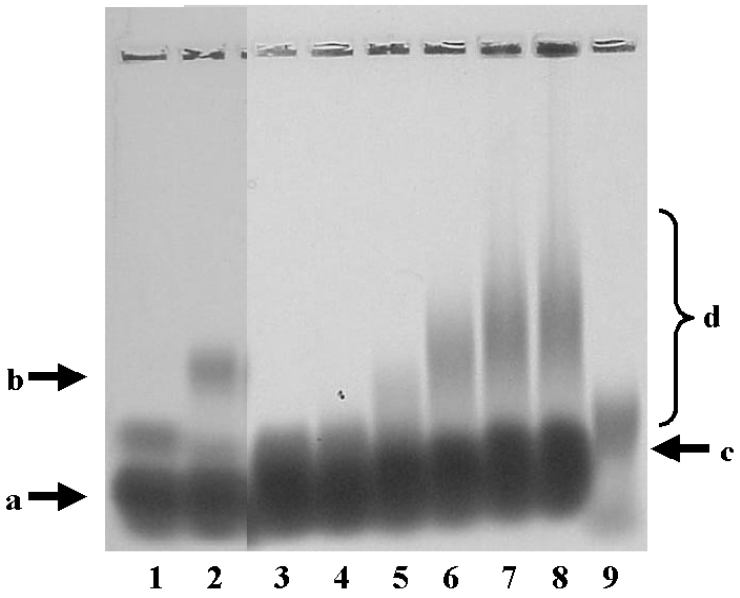

Figure 3.3: SDS gel electrophoresis of unheated milk (lane 2), unheated milk with DTT (lane 1), the pellet of unheated milk (lane 3) and the pellet of milk heated at 70, 75, 80, 85 and 90°C (10 min; lanes 4 to 8 respectively) obtained by renneting and centrifugation and dissolved in SDS-buffer. Lane 9 contains whey protein aggregates with a hydrodynamic diameter of 62 nm. Indicated in the gels are the bands corresponding to casein monomers and native whey proteins (arrow [a]), the casein polymers in unheated milk before and after renneting (arrow [b] and [c], respectively) and the casein-whey protein polymers formed during heat treatment (bracket [d]).

literature (29). Renneting of unheated milk causes a clear decrease in size of the casein polymers, which is likely due to rennet hydrolysing κ-casein into a casein macropeptide (CMP) and para-κ-casein, with sizes of 6.8 and 12.3 kDa, respectively (18, 31, 43). The CMP is released into the serum while the para-κ-casein remains disulfide-linked to the casein micelle. Summarising, we can conclude that casein micelles are not purely association colloids composed of individual caseins, but that disulfide bonds between the caseins contribute significantly to the integrity of the casein micelle.

Heated milk

Heat treatment of milk (Figure 3.3, lanes 4 to 8) affects the electrophoretic mobility of the protein structures, inducing slower-migrating protein structures with increasing temperature of heat treatment (Figure 3.3, bracket [d]). The change in electrophoretic mobility is due to disulfide interactions between whey proteins and κ-casein (16). It is possible that α_{s2}-casein also reacts with whey proteins due to its cysteine residue, but this has not yet been reported in the literature and was not studied in this paper. As almost all κ-casein is present in large polymers (4, 29, 36, 37) the disulfide interactions with whey proteins will mainly affect the polymeric protein structure (Figure 3.3, arrow c), which explains the very large protein structures formed during heat treatment. Summarising, heat treatment of milk causes whey protein association with κ-casein present in polymers which results in very large protein structures. The results do not provide information on the distribution of the whey proteins on the surface of the casein micelle. This will be the subject of chapter 4 (41).

Acid-induced gelation

By addition of GDL milk was slowly acidified and the mobility of the particles was monitored by DWS, represented by $\tau_{1/2}$. Figure 3.4 shows the $\tau_{1/2}$ acidification curves of unheated milk and milk heated at 70, 75, 80, 85 and 90°C. Unheated milk shows a steady decrease of $\tau_{1/2}$ starting at pH 5.3 and reaching a minimum at pH 5.0, followed by a sharp increase. The start of this increase is hereafter called the gelation pH. Milk heated at 70°C shows a similar behaviour to unheated milk, while milk heated at 75°C still has a minimum in $\tau_{1/2}$ before the onset of gelation but a slightly higher gelation pH. Milk heated at 80°C and higher temperature has much higher gelation pH values and a steady decrease prior to the gelation pH is no longer observed.

Increasing heating temperature causes a shift to higher gelation pH. This is caused by the whey protein coating of the casein micelles and the presence of whey protein aggregates. The whey protein coating changes the surface properties of the casein micelle due to changes in the isoelectric pH, i.e 4.6 for casein micelle to 5.2 for β-lg, which causes a decrease in electrostatic repulsion at higher pH values and an earlier start of gelation (15, 23, 39). The minimum in $\tau_{1/2}$ present in unheated milk is due to the collapse of the hairy brush of κ-casein molecules at approaching the pH at which the

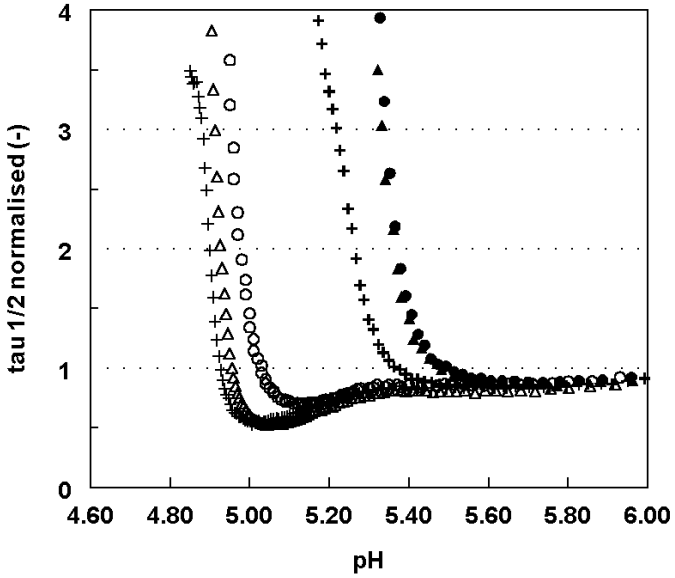

Figure 3.4: Acidification traces of skim milk ($\tau_{1/2}$ versus pH) unheated (+) and heated at 70 (△), 75 (○), 80 (✚), 85 (▲) and 90°C (●) as monitored by DWS.

micelles tend to neutrality (19) and also to changes in the micel due to release of calcium phosphate and serum caseins. In milk heated at 80, 85 and 90°C the gelation has started already at pH values of 5.4 and higher, while the destabilisation of the κ-casein brush started at pH 5.3 (unheated milk) and therefore the minimum is no longer observed.

In Figure 3.5 the gelation pH is plotted versus the percentage of denatured β-lg, the percentage of β-lg present in soluble aggregates and the percentage of β-lg associated with the casein micelles as determined in milk heated at temperatures ranging from 70 to 90°C. In all cases the data show a good linear correlation with the gelation pH.

The pH of gelation is mainly determined by the amount of denatured β-lg, as was demonstrated by Vasbinder et al. (39). Therefore, only the percentage of denatured β-lg was plotted as a function of the gelation pH. It appears that the gelation pH correlates linearly with the percentage of denatured β-lg present in heated milk. The total amount of denatured β-lg is composed of aggregates and protein associated with the casein micelle, which are present in a constant ratio in the milks heated at pH 6.7 at 70 to 90°C. Therefore, under these conditions it is not possible to separate the effects of the two groups of denatured whey proteins. The linear correlation observed in Figure 3.5 demonstrates that the changed gelation

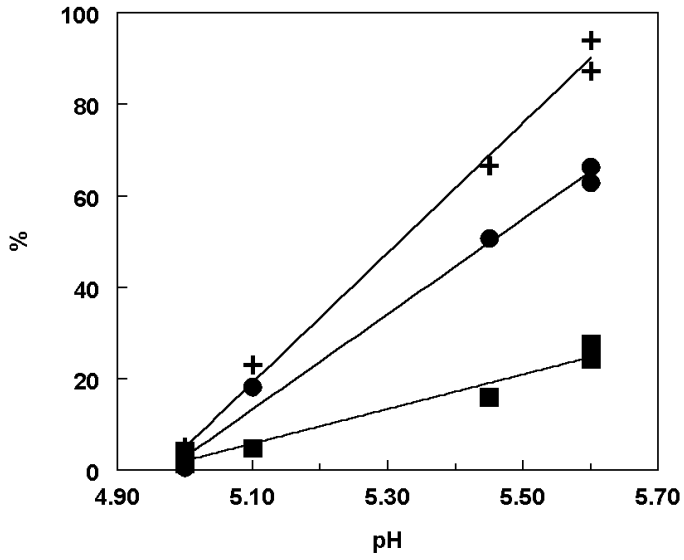

Figure 3.5: Gelation pH versus percentage of total denatured β-lg (✚), β-lg associated with the casein micelle (●) and β-lg in whey protein aggregates (■) present in skim milk unheated and heated at 70 to 90°C (10 min).

properties of heated milk are completely caused by whey protein denaturation. If the ratio between aggregates and associated whey proteins is constant, the total amount of denatured protein determines the gelation properties.

Conclusions

Heat treatment of milk at temperatures ranging from 70 to 90°C causes denaturation of whey proteins of which β-lg denatures at a higher rate than α-lac. The denatured whey proteins form soluble whey protein aggregates and whey protein coating of the casein micelles. At 90°C aggregates are formed with a size of 60-100 nm containing about 25% of both whey proteins, and about 65% of β-lg and 50% of α-lac has associated with the casein micelle. The aggregates show a ratio of α-lac to β-lg which is representative of the ratio in total denaturated whey proteins in milk. The whey protein coating on the casein micelle is enriched in β-lg. Since κ-casein in milk is present in large polymeric structures, heat-induced association of κ-casein and whey proteins causes very large polymeric protein structures. Heat treatment of milk causes a shift to higher gelation pH values upon acidification. These gelation pH values correlate linearly with the total amount of denatured whey proteins, the amount of soluble aggregates and the amount of whey proteins associated with the casein micelle. It is expected that the whey protein distribution affects the gelation properties; this will be the subject of chapter 4 (41).

References

1. Alting, A.C., R.J. Hamer, C.G. de Kruif and R.W. Visschers. 2000. Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. J. Agric. Food Chem. 48: 5001.
2. Anema, S.G., 2000. Effect of milk concentration on the irreversible thermal denaturation and disulfide aggregation of β-lactoglobulin. J. Agric. Food Chem, 48: 4168.
3. Anema, S.G., 2001. Kinetics of the irreversible thermal denaturation and disulfide aggregation of α-lactalbumin in milk samples of various concentrations. J. Food Sci. 66: 1: 2.
4. Annan, W.D. and W. Manson. 1969. A fractionation of the αs-casein complex of bovine milk. J. Dairy Res. 36: 259.
5. Barbut, S. and E.A. Foegeding. 1993. Ca2+-induced gelation of pre-heated whey protein isolate. J. Food Sc. 58: 4: 867.

6. Corredig, M. and D.G. Dalgleish. 1996. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Res. Int. 29: 1: 49.
7. Dannenberg, F. and H.G. Kessler. 1988. Reaction kinetics of the denaturation of whey proteins in milk. J. Food Sci.53: 258.
8. Dannenberg, H. and H.G. Kessler. 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 1. Syneresis. Milchwissenschaft. 43: 632.
9. Dannenberg, F. and H.G. Kessler 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 2. Firmness and flow properties. Milchwissenschaft. 43: 700.
10. Dalgleish, D.G., L. van Mourik, M. Corredig. 1997. Heat-induced interactions of whey proteins and casein micelles with different concentrations of α-lactalbumin and β-lactoglobulin. J. Agric. Food Chem. 45: 4806.
11. Heertje, I., J. Visser and P. Smits. 1985. Structure formation in acid milk gels. Food Microstructure. 4: 267.
12. Hill, A.R. 1989. The β-lactoglobulin-k-casein complex. Can. Inst. Food Sci. Technol. J. 22: 2: 120.
13. Holt, C., A.M. Kimber, B. Brooker and J.H. Prentice. 1978. Average radii of bovine casein micelles. XX-International-Dairy-Congress; E, 234.
14. Hongsprabhas, P. ans S. Barbut. 1997. Protein and salt effects on Ca^{2+}-induced cold gelation of whey protein isolate. J. Food Sci. 62: 2: 382.
15. Horne, D.S. and C.M. Davidson. 1993. Influence of heat treatment on gel formation in acidified milks. Protein and fat globule modification, in Proceedings of IDF Seminar. 267.
16. Jang, H.D. and H.E. Swaisgood. 1990. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. J. Dairy Sci. 73: 900.
17. de Kruif, C.G., M.A.M. Hoffmann, M.E. van Marle, P.J.J.M. van Mil, S.P.F.M. Roefs, M. Verheul and P. Zoon. 1995. Gelation of proteins from milk. Faraday Discuss. 101: 185.
18. de Kruif, C.G. and E.B. Zhulina. 1996. κ-casein as a polyelectrolyte brush on the surface of casein micelles. Colloids and Surfaces A. 117: 151.
19. de Kruif, C.G. 1997. Skim milk acidification. J. Colloids Interface Sci. 185: 19.
20. Law, A.J.R., D.S. Horne, J.M. Banks and J. Leaver. 1994. Heat-induced changes in the whey proteins and caseins. Milchwissenschaft. 49: 3: 125.
21. Law, A.J.R. and J. Leaver. 1997. Effect of milk protein concentration on the rates of thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 45: 4255.
22. Law, A.J.R. and J. Leaver. 2000. Effect of pH on the thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 48: 672.
23. Lucey, J.A., C. Tet Teo, P.A. Munro and H. Singh. 1997. Rheological properties at smal1 (dynamic) and large (yield) deformations of acid gels made from heated milk. J. Dairy Res. 64: 591.
24. Lucey, J.A., P.A. Munro and H. Singh. 1998. Rheological properties and microstructure of acid milk gels as affected by fat content and heat treatment. J. Food Sci. 63: 660.

25. Noh, B., T. Richardson and L.K. Creamer. 1989. Radiolabelling study of the heat-induced interactions between α-lactalbumin, β-lactoglobulin and κ-casein in milk and in buffer solutions. J. Food Sci. 54: 4: 889.
26. Oldfield, D.J., H. Singh and M.W. Taylor. 1998. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. Int. Dairy J. 8: 765.
27. Oldfield, D.J., H. Singh, M.W. Taylor, K.N. Pearce. 2000. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk, Int. Dairy J. 10: 509.
28. Parnell-Clunies, E., Y. Kakuda, J.M. deMan and F. Cazzola. 1988. Gelation profiles of yoghurt as affected by heat treatment of milk. J. Dairy Sci. 71: 582.
29. Rasmussen, L.K. and T.E. Petersen. 1991. Purification of disulfide-linked α_{s2}- and κ-casein from bovine milk. J. Dairy Res. 58: 187.
30. Roefs, S.P.F.M., C.G. de Kruif. 1994. A model for the denaturation and aggregation of β-lactoglobulin. Eur. J. Biochem. 226: 883.
31. Rollema, H.S. Casein Association and micelle formation, in Advanced Dairy Chemistry, volume 1. Proteins, edited by P.F. Fox, Elsevier Science Publishers, England. 111.
32. Schmidt, D.G., P. Walstra and W. Bucheim. 1973. The size distribution of casein micelles in cow's milk. Neth. Milk Dairy J. 27: 128
33. Singh, H.S. 1993. Heat-induced interactions of proteins in milk. Protein and fat globule modifications-IDF seminar, special issue 9303. 191.
34. Singh, H., M.S. Roberts, P.A. Munro and C.T. Teo. 1996. Acid-induced dissociation of casein micelles in milk: effect of heat treatment. J. Dairy Sci. 79: 1340.
35. Smits, P. and J.H. van Brouwershaven. 1980. Heat induced association of β-lactoglobulin and casein micelles. J. Dairy Res. 47: 313.
36. Swaisgood, H.E., J.R. Brunner and H.A. Lillevik. 1964. Physical parameters of κ-casein from cow's milk. Biochemistry. 3: 1616.
37. Talbot, B. and D.F. Waugh. 1970. Micelle forming characteristics of monomeric and covalent polymeric κ-caseins. Biochemistry. 9: 2807.
38. Tuinier, R. An exocellular polysaccharide and its interaction with proteins. Ph.D. Diss. University of Wageningen, Wageningen, Neth.
39. Vasbinder, A.J., P.J.J.M. van Mil, A. Bot and C.G. de Kruif. 2001. Acid-induced gelation of heat treated milk studied by Diffusing Wave Spectroscopy. Colloids and Surfaces B. 21: 245. Chapter 6 in this thesis.
40. Vasbinder, A.J., A.C. Alting, R.W. Visschers and C.G. de Kruif. Texture of acid milk gels: formation of disulfide cross-links during acidification, submitted for publication in the Int. Dairy J. Chapter 7 in this thesis.
41. Vasbinder, A.J. and C.G. de Kruif. Casein-whey protein interactions in milk heated at pH 6.3-6.9. manuscript in preparation. Chapter 4 in this thesis.
42. van Vliet, T. and C.J.A.M. Keetels 1995. Effect of preheating of milk on the structure of acidified milk gels. Neth. Milk Dairy J. 49: 27.
43. Walstra, P. and R. Jenness. 1984. Dairy Chemistry and Physics, John Wiley and sons, Inc, USA.

Chapter 4: Casein-whey protein interactions in milk heated at pH 6.35 - 6.9

Abstract

Heat-treatment of milk causes denaturation of whey proteins, leading to a complex mixture of whey protein aggregates and whey protein coated casein micelles. In this chapter we studied the effect of pH-adjustment of milk (pH range 6.9 to 6.35) prior to heat-treatment (10 min at 80°C) on the distribution of denatured whey proteins in aggregates and coating and the homogeneity of the whey protein coating on the casein micelles. After heat treatment at pH 6.9 most whey proteins are present in soluble whey protein aggregates while heating at pH 6.55 and lower causes association of all whey proteins with the casein micelle. Heating of milk at pH 6.35 causes a clearly more inhomogeneous coating than heating at pH 6.55. This pH-dependent whey protein denaturation is schematically depicted in a model.

The pH-dependent whey protein denaturation and aggregation correlates to changes in acid- and rennet-induced gelation properties. Heating at the pH 6.9 decreased the rate of rennet-induced flocculation compared to unheated milk, whereas the rate for samples heated at the pH 6.35 were quite comparable to those for unheated milk. The behaviour could be related to the inhomogeneity of the whey protein coating of the casein micelle when heated at low pH. Decreasing the pH of milk prior to heat treatment causes a decrease in the acid-induced gelation pH which could be related to formation of soluble whey protein aggregates and the inhomogeneity of the whey protein coating of the casein micelles. This chapter extends the current knowledge on the relation between pH-induced denaturation and acid- and rennet-induced gelation properties.

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Submitted for publication

Introduction

Milk is heated to improve its keeping quality by deactivating microorganisms. In addition, heating leads to desirable properties in the final product, like an increased viscosity of yoghurt (23). No noticeable heat-induced effects are observed on the structure of the casein micelle fraction in the temperature range 70-100°C (11). However, heating does have a definite effect on the (bovine) whey protein fraction of milk, which consists mainly of β-lactoglobulin (β-lg) and α-lactalbumin (α-lac). Upon heating, a reactive thiol group is exposed in β-lg due to conformational changes of the molecule. This reactive thiol group can form disulfide links with other proteins having a reactive thiol groups or through thiol group-disulfide bridge exchange reactions. The reaction makes the denaturation process irreversible, in contrast to the reversible denaturation of porcine β-lg which lacks free thiol groups (3, 7, 27). The process of denaturation and subsequent aggregation of bovine β-lg resembles a polymerisation process, in which the unfolding step represents the initiation (10, 21). α-Lac cannot initiate the polymerisation process due to the absence of a free thiol group; however as it has four disulfide bridges it is irreversibly denatured in the presence of β-lg due to thiol group-disulfide bridge exchange reactions. β-Lg also exhibits interactions with the casein micelles through thiol group-disulfide bridge exchange reactions with κ- and α_{s2}-casein. The initial step of this process is believed to be physical in nature, but the final interaction is covalent, i.e. disulfide linked (6, 9). So after heating, the hairy brush of the casein micelles contains associated whey proteins. However, not all β-lg and α-lac is bound to the casein micelle. A considerable fraction forms pure whey protein aggregates, a feature often not recognised in the literature (28). Summarising, heat treatment of milk results in a complex mixture of native whey proteins, whey protein aggregates and casein micelles covered with appendages of whey protein.

In the literature it is shown that the final composition of heated milk consisting of casein micelles and denatured whey protein aggregates, depends on the pH and temperature of heat treatment (2, 4, 5, 12-17, 28). At higher temperatures more denaturation of whey proteins occurs (5) and both formation of aggregates and coating of the casein micelles take place at a constant ratio for heat treatment of

70 to 90°C (28). By varying the pH between 6 and 7 prior to heat treatment the mechanism of denaturation is influenced. The total degree of denaturation is rather constant (12), but heat treatment at higher pH results in the formation of more whey protein aggregates while heat treatment at lower pH results in more association of the whey proteins with the casein micelle (2, 4, 16). In this paper we propose a model for whey protein denaturation and the subsequent association with casein micelles as a function of the pH of heat treatment. As such this has not been investigated before, but it appears to be very relevant to explain changes in rennet- and acid-induced gel formation as a function of the pH at which heating takes place. Singh et al. (24) reported a threefold decrease in rennet clotting time when the pH at which heating occurs was decreased from 7.0 to 6.5. However, no explanation was given for this phenomenon. As far as we know the acid-induced gelation as function of pH at which heat treatment is performed has not been studied until now. In this paper we relate the model, showing the distribution of denatured whey proteins in aggregates and coating of the micelle and the type of coating, to the changed rennet- and acid-induced gelation properties. The pH values used in this study ranged from 6.35 to 6.9. The applied changes in pH are rather small but appear to have large effects on the whey protein denaturation and subsequently on the gelation properties, indicating the relevance of controlling the pH during processing of milk. Our findings can also provide opportunities to design new products based on acidification and renneting of milk.

Materials and Methods

Skim milk

Skim milk heated for 10 seconds at 72°C was obtained from the pilot plant at NIZO food research, Ede. Sodium azide (0.02% w/w) was added to prevent bacterial growth and the milk was stored at 4°C.

Whey Protein Isolate solution

Whey Protein Isolate (Bipro; Davisco Food International Inc., USA; for composition see materials and methods chapter 3) solution was prepared at a concentration of 9% (w/w). The solution was stirred for 2 hours at room temperature and subsequently filtered though a 0.45μm filter; 0.02% (w/w) NaN_3 was then added. Heating this

solution for 2 hours at 68.5°C led to the formation of aggregates with a size of 62 nm which was determined by Dynamic Light Scattering as described in Vasbinder et al. (29).

pH adjustment

Cold milk was equilibrated at room temperature for 2 hours. The milk was acidified by addition of 0, 0.05, 0.10 and 0.15% (w/w) glucono-delta-lactone (GDL) in order to reach a final pH of 6.7 to 6.35 after overnight incubation at 20°C. pH 6.9 was obtained by drop-wise addition of 0.5M NaOH under stirring.

Heat treatment of milk

Glass tubes (volume 8 ml, diameter 1 cm) were filled with 5 ml of milk and heated for 10 min in a water-bath at 80°C, then cooled with tap water to room temperature.

Fractionation of whey proteins

Acetic acid precipitation

An amount of 0.4 g of milk was mixed with 0.8 g of distilled water (40°C) and 40 µl acetic acid (10%) in an Eppendorf tube (2 ml). After mixing (vortex) and 10 min waiting, 40 µl of sodium acetate (1M) and 0.72 g of distilled water were added and the solution was mixed again. After 1 hour standing the solution was centrifuged for 5 min at 3000g. The supernatant containing native whey proteins was analysed by capillary electrophoresis.

Rennet precipitation

Renneting of the milks was performed essentially based on the method of Noh and Richardson (1989), but without addition of calcium chloride. The supernatant containing native whey proteins and soluble aggregates was analysed by capillary electrophoresis.

Capillary electrophoresis (CE)

The samples were analysed by CE (Beckman P/Ace 5000; Beckman Coulter Inc.) with a capillary (Agilent, µSil-wax, internal diameter 0.05 mm) of 60 cm length. The samples were injected during 20 seconds with a pressure of 0.5 psi. The electrophoresis was carried

out at 45°C using a voltage of 25 kV towards the cathode and detection was at 214 nm. The experiments were performed in buffer containing 6M urea and dithiothreitol.

Preparation of SDS gel electrophoresis samples

The rennet-induced supernatant and the milks were diluted 1 to 1 with 20 mM Bis-Tris buffer and 5% sodium dodecyl sulphate (SDS) (pH 7.0) (1); the rennet-induced precipitate was solubilized by replacing the supernatant with an equal volume of the Bis-Tris buffer. All samples were held overnight at ambient temperature; the precipitates in buffer were constantly stirred. In some experiments a treatment with the disulfide-reducing agent dithiothreitol (DTT) (0.05%) was carried out to reduce the disulfide bonds in solubilized samples.

Agarose gel electrophoresis

SDS-agarose gels were prepared at a concentration of 0.4% agarose. Per sample 20µl was applied on the gel; in the case of aggregates the sample volume was 100µl. The agarose gel electrophoresis was carried out according to Alting et al. (1). Briefly the electrophoresis buffer consisted of 100 mM Tris, 50 mM sodium acetate, 2 mM EDTA and 0.1% SDS, and was adjusted to pH 7.9 with concentrated acetic acid. Prior to electrophoresis, 5% of a solution containing 60% glycerol and 0.002% bromophenol blue was added to the samples stirred overnight. The gels were run with a constant voltage of 50V for approximately 2 hours and finally stained with Phastgel blue R.

Diffusing wave spectroscopy (DWS) measurement of rennet and acid-induced gelation of milk

Rennet-induced gelation was initiated by incubating the heat-treated milk for 75 min at 30°C, followed by addition of 500-fold diluted rennet to a final concentration of 0.005% in the milk. Acid-induced gelation was started by incubating the milk for 75 min at 32°C, followed by addition of 1.2% GDL to the milk. The clotting of the milk was monitored by DWS. Light from a 5 mW He-Ne laser (632.8 nm) was passed through a multi-mode fibre into the milk. The back-scattered light was monitored by a single-mode fibre located at 3.0 mm from the input fibre. The scattered light was detected with a

Photo Multiplier Tube (ALV SO-SIPD and fed to a PC interfaced autocorrelator (Flex 5000, correlator.com). The correlator calculates the autocorrelation function in real time. The time at which the autocorrelation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{½}$ (30). The correlation functions were monitored in time at intervals of 2 min. All data were normalised by the $\tau_{½}$ value (average of five measurement) of the same sample prior to rennet or GDL addition. This eliminates slight variations between the samples and fibres.

Results and Discussion

Whey protein denaturation

Renneting of milk causes degradation of κ-casein resulting in flocculation of the casein micelles, fractionating the whey proteins in soluble aggregates and native protein in the supernatant and the whey proteins associated with the casein micelles in the pellet. With acid-induced precipitation of the milk only native whey proteins remain in the serum and all denatured whey proteins precipitate in the pellet. Figure 4.1 shows the percentages of α-lac (Figure 4.1a) and β-lg (Figure 4.1b) present as native, in aggregates and as coating of the micelle as function of the pH at which heat treatment is applied. A rather constant denaturation is observed at pH 6.45, 6.55 and 6.7, i.e. 40% of α-lac and 70% of β-lg is denatured. Heat treatment at pH 6.9 causes a slight increase in α-lac denaturation, while β-lg shows more denaturation at pH 6.35. In contrast to the rather constant degree of denaturation, the distribution of the whey proteins in soluble aggregates and association with the casein micelle shows a very pH dependent behaviour. Higher pH values cause formation of soluble aggregates, while at lower pH all whey proteins have associated with the micelle. This behaviour is similar for both whey proteins.

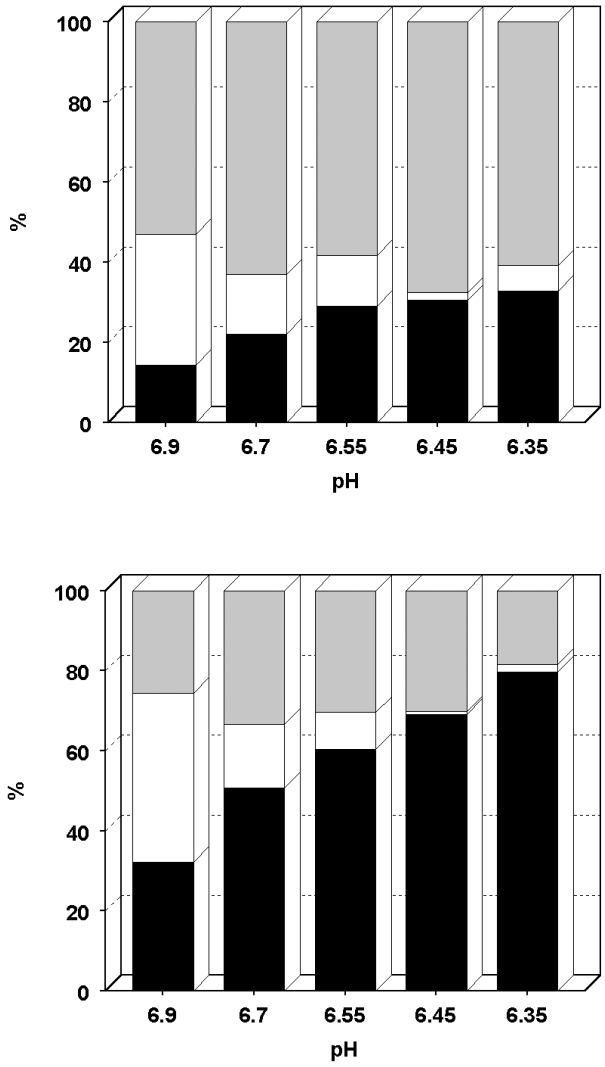

Figure 4.1: Degree of denaturation of α-lac (upper graph) and β-lg (lower graph) after heat treatment of 10 min at 80°C as a function of the pH of milk (pH 6.35-6.9). The whey proteins associated with the casein micelle are represented by the black bars, the whey protein aggregates by the white bars and native whey proteins by grey bars.

The higher denaturation rate of β-lg and the degrees of denaturation in the pH range of 6.35 to 6.9 are in agreement with work of Law and Leaver (12). The supernatant obtained by renneting contained whey proteins and some casein, which was less than 3% of total casein. The caseins involved in the thiol group-disulfide bridge exchange reactions with β-lg, i.e. α_{s2} and κ-casein, could hardly be detected in the serum. Therefore the aggregates in the serum will further henceforth be regarded as whey protein aggregates. In chapter 3 (28) the fractionation technique based on renneting is more extensively discussed and compared to fractionation by using ultracentrifugation. Heat treatment at higher pH causes a clear

formation of whey protein aggregates, indicating a pH dependent aggregation mechanism. Anema and Klostermeyer (2) demonstrated using ultracentrifugation that at higher pH more whey proteins remained soluble than at lower pH: respectively 60% of β-lg at pH 6.9 and 15% of β-lg at pH 6.35 after heat treatment of 15 min at 80°C. These results are in line with the sum of native and soluble whey protein aggregates observed in this study. Heat treatment at lower pH resulted in a clear increase in association of whey proteins with the casein micelles, which was also observed by Oldfield et al. (17) by using ultracentrifugation. They observed an association of 90, 80 and 60% at pH 6.48, 6.60 and 6.83, respectively (10 min at 90°C), which demonstrates a similar trend as observed in this paper. Summarising, heat treatment of milk in the pH range of 6.35 to 6.9 causes a rather constant denaturation of whey proteins, but at lower pH all whey proteins associate with the micelle, while at higher pH a significant amount of soluble whey protein aggregates is formed.

Whey protein aggregates: size and quantity

The supernatant obtained by rennet treatment of milk heated at pH 6.9 to 6.35 for 10 min at 80°C was characterised by SDS-agarose gel electrophoresis (Figure 4.2) . In milk heated at pH 6.9 and 6.7 aggregates are observed. At the lower pH of heat treatment no aggregates could be detected. The molecular weight and thereby the

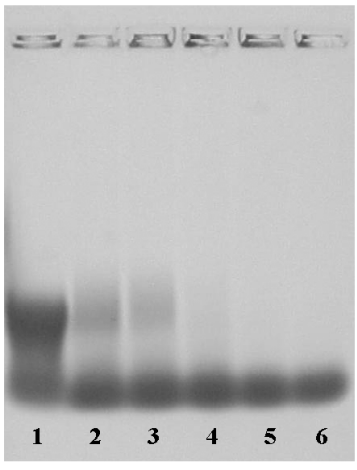

Figure 4.2: SDS agarose gel electrophoresis of the supernatants obtained by rennet treatment of milk heated for 10 min at 80°C as a function of the pH at which heating is performed, i.e pH 6.9, 6.7, 6.55, 6.45 and 6.35 in lanes 2 to 6, respectively. Only covalent interactions remain intact as the supernatants are diluted with SDS-buffer. Lane 1 contains a marker with whey protein aggregates of 62 nm.

size of the aggregates formed during heat treatment were estimated by using a marker consisting of whey protein aggregates (diameter 62 nm) prepared from whey protein isolate. The aggregates formed have molecular weights similar to and slightly larger than the marker, indicating sizes of 60 to 100 nm. No effect of pH was observed on the molecular weight of the aggregates. The band at pH 6.9 is stained darker, indicating a higher concentration of aggregates.

The results obtained by SDS-agarose gel electrophoresis are in agreement with the quantification as presented in Figure 4.1. Only in milk heated at pH 6.7 and 6.9 aggregates could be detected. It is remarkable that heat treatment at pH 6.9 only results in the formation of more aggregates and causes no increase in size of the aggregates.

Whey protein coated casein micelles: distribution of the whey proteins

Figure 4.3 shows SDS gel electrophoresis patterns of the pellets obtained by rennet treatment of milk heated at pH 6.9 to 6.35 (10 min at 80°C) after redispersing in SDS-buffer, by which non-covalent bonds are disrupted. Lane 2, unheated milk, shows a pattern of two protein bands. The band with the lower mobility (arrow [a]) is increased in mobility by applying DTT, which disrupts disulfide-linked structures (arrow [b], lane 1). Lane 3 containing the redissolved pellet of renneted unheated milk shows one broad protein band. Lanes 4 and 5, representing the rennet-induced pellet of milk heated at pH 6.9 and 6.7, contain a band with a decreased mobility compared to unheated, renneted milk (lane 3). At pH 6.55 (lane 6) the mobility of this band has decreased slightly further, but clear changes in the pattern begin to occur at pH 6.45 and 6.35 (lanes 7 and 8). Heat treatment at lower pH values causes formation of bands with a lower electrophoretic mobility compared to milk heated at pH 6.7 and 6.9.

Casein micelles consist of mainly non-covalently bound caseins and are almost completely disrupted in the presence of SDS. Only the disulfide-linked structures consisting of κ- and α_{s2}-casein remain intact (lane 1 in Figure 4.3; 19, 20). These structures cause the protein band indicated by arrow [a]. Addition of DTT to the sample, which disrupts disulfide bonds, causes an increase in mobility, indicating disruption to smaller fragments (28). The pattern with two protein bands remains intact in the pellets of milk heated at pH 6.7

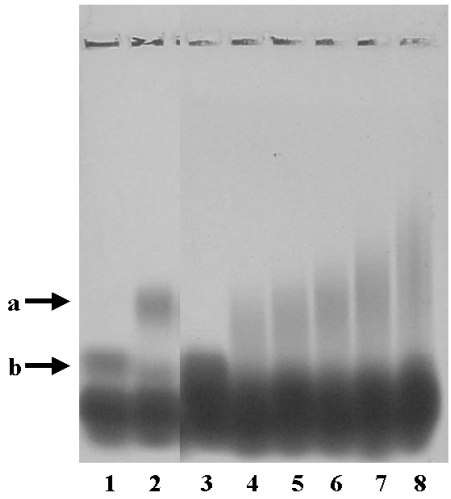

Figure 4.3: SDS agarose gel electrophoresis of the pellets obtained by rennet treatment of milk heated for 10 min at 80°C as a function of the pH at which heating is performed, i.e. pH 6.9, 6.7, 6.55, 6.45 and 6.35 in lanes 4-8, respectively. Only covalent interactions remain intact after dispersal of the pellets in SDS-buffer. Lanes 1 to 3 contain, respectively, unheated milk with DTT, unheated milk and the redissolved pellet of renneted unheated milk. The arrows indicate relevant structures which are discussed in the text.

and 6.9, although the band containing the larger particles increases in size compared to unheated milk. The increase in size is due to a covalent association of denatured whey proteins with disulfide-linked casein polymers. Milk heated at pH 6.55, 6.45 or 6.35 has a comparable degree of denaturation to pH 6.9 and 6.7. However, in these milks all whey proteins are associated with the casein micelle. Therefore, the larger structures formed by heating milk at lower pH, e.g. 6.35 compared to 6.55, cannot be explained by association of more whey proteins on the micelle and therefore indicate a different aggregation mechanism of whey proteins with the casein micelle at pH 6.35 than at 6.55. The larger structures observed indicate that heating at lower pH causes more inhomogeneous coating of the micelles. In milk β-lg and κ-casein are present in equal molar concentrations. The denaturation of β-lg in all milk samples investigated is about 70%, so in theory only 70% of all κ-caseins can react with one molecule of β-lg creating not even a complete monolayer. Under these conditions 30% of all κ-casein molecules remain unreacted and this number increases when more whey proteins associate with one κ-casein molecule which occurs by heating at lower pH. Heat treatment at pH 6.9 to 6.55 increases the amount of β-lg associated with the casein micelle from 30 to 60%, while only a very

slight decrease in mobility of the protein structures on the gel is observed. This demonstrates that a rather homogeneous coating of the casein micelles takes place when the milk is heated at higher pH values.

Model

Figure 4.4 shows a schematic representation of the interactions of casein micelles and whey proteins in milk heated in the pH range of 6.9 to 6.35. At higher pH a rather homogeneous coating of the casein micelles and formation of large whey protein aggregates occurs. At pH 6.55 no aggregates are formed, but the type of coating of the casein micelles is probably comparable to that occurring at higher pH of heating. At lower pH no separate aggregates are formed, but cluster of whey proteins associate with the casein micelle.

Due to the slight pH changes induced in the milk prior to heat treatment large effects are observed in the aggregation mechanism of whey proteins. At high pH β-lg-β-lg interactions causing whey protein aggregates are favoured over κ-casein-β-lg interactions, while κ-casein-β-lg-β-lg reactions hardly take place. At lower pH, formation of separate whey protein aggregates hardly occurs, but clusters of whey proteins are formed on the surface of the casein micelle, indicating that in spite of the equal molarity of κ-casein and β-lg in milk a considerable number of κ-casein molecules are not involved in the process. Apparently, at these conditions κ-casein-$(\beta\text{-lg})_n$ interactions are favoured over κ-casein-β-lg interactions. These results

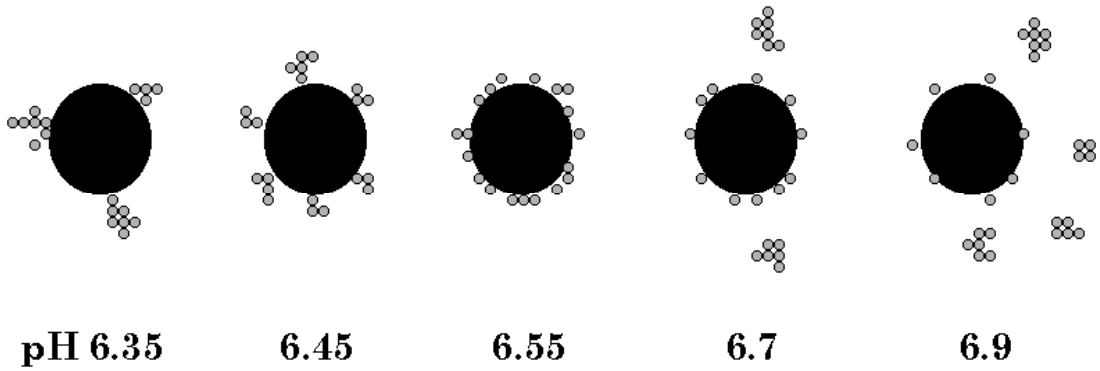

Figure 4.4: A schematic representation of the interactions between casein micelles and whey proteins occurring in milk during heat treatment for 10 min at 80°C at pH values ranging from 6.35 to 6.9. The small circles represent denatured whey proteins, the large circles the casein micelles. The whey proteins are either present in aggregates or covalently associated with the casein micelle. Native whey proteins are not included in the figure.

demonstrate that the reactivity of κ-casein and β-lg is very pH dependent. It is known from other workers (8, 18) that slight pH changes cause a strong change in reaction kinetics of whey proteins. The formation of aggregates at higher pH values is not attributed to the release of κ-casein-β-lg complexes as is sometimes suggested in the literature (17). Hardly any casein was present in the supernatant and it is shown that micellar κ-casein mainly reacts with a small number of whey proteins. Release of this complex would probably not affect the reactivity of the complex and the results in this work clearly indicate that no large structures are formed on the surface of the micelle. Therefore, we conclude that formation of soluble aggregates takes place in solution.

Rennet-induced gelation of heated milk

Rennet-induced gelation of heated milk was monitored by DWS measurements. On gelation the measured relaxation time ($\tau_{½}$) sharply increases. The parameter $\tau_{½}$ reflects the mobility of the particles in solution, i.e. the casein micelles in milk, and is therefore a sensitive parameter for monitoring the clotting of the micelles and the beginning of the subsequent gelation. Figure 4.5 shows the rennet-induced gelation curves of milk heated at pH 6.9 to 6.35 (Figure 4.5a) and of unheated milk, unheated milk with extra native whey protein, unheated milk with whey protein aggregates and milk heated at pH 6.7 subjected to a pH-cycling to pH 6.35 (Figure 4.5b). The traces of all unheated milks show identical behaviour, i.e. a slight decrease in $\tau_{½}$ in the first 200 min of incubation and a start of clotting after about 180 min with a sharp rise of the $\tau_{½}$. Renneting of milk heated at pH 6.7 demonstrated the same minimum and start of flocculation (after 200 min) as unheated milk, but the flocculation proceeded at a much lower rate. Subjecting the heated milk to pH-cycling (6.7 to 6.35 to 6.7) prior to renneting led to a slightly improved clotting behaviour compared to the non-pH-cycled sample, but it still flocculated rather poorly. Milk heated at pH 6.55 flocculated even more slowly, while milk heated at pH 6.9 and 6.45 flocculated slightly better. Milk heated at pH 6.35 showed a strongly improved flocculation compared to the other heated milks.

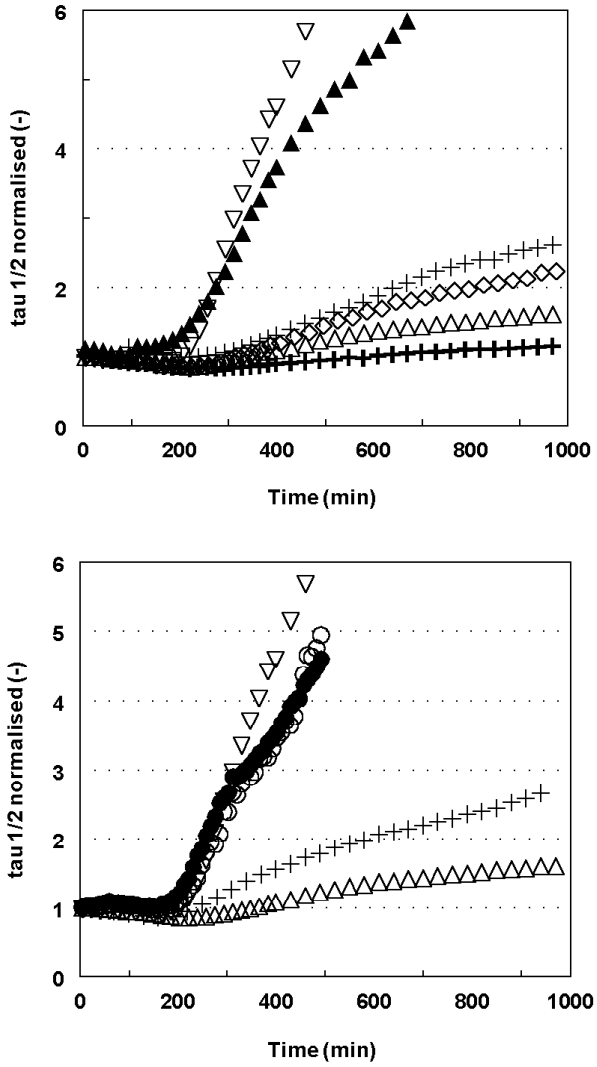

Figure 4.5: $\tau_{½}$ as function of renneting time obtained by DWS of milk heated for 10 min at 80°C at a pH of 6.9 (+), 6.7 (△), 6.55 (✚), 6.45 (◇), 6.35 (▲) and unheated milk (▽) (Figure 4.5a; upper graph) and of unheated milk (▽), unheated milk with 0.44% WPI (●), unheated milk with 0.44% WPI aggregates (○), milk heated for 10 min at 80°C (△; pH 6.7) and milk heated for 10 min at 80°C (pH 6.7) subjected to pH cycling to 6.35 and 6.7 (✚) (Figure 4.5b; lower graph). All samples were renneted at pH 6.7 with 0.005% rennet at 30°C.

It is generally known that heat treatment deteriorates the curd-forming properties of milk (25, 31). Singh et al. (24) observed a decreased flocculation as the pH at which heat treatment was performed was increased from 6.5 to 7. This is not in agreement with our results, as the lowest rate of flocculation was observed at pH 6.55. These differences might be due to the different pH of renneting (6.5 versus 6.7) or to the different analytical tool used to determine flocculation, i.e. the visually determined rennet clotting time (24) and

the rate of flocculation studied by DWS. There are various possible explanations for the effect of the pH at which heating is carried out on the flocculation speed, i.e. changes in CMP release, pH cycling and whey protein coating of the casein micelles. Differences in CMP release cannot explain the observed effects as no changes in the enzymatic cleavage of the κ-casein in these milks was observed (results not shown). Only a slight increase in rate of flocculation was observed when milk was heated at pH 6.7 and subjected to pH cycling (6.7-6.35-6.7). This is in agreement with the literature (13, 24), but this effect is not sufficient to explain the increased rate of flocculation of milk heated at pH 6.35. Denaturation of whey proteins causes a decreased rate of clotting (25). There are two groups of denatured whey proteins, i.e. the whey protein aggregates and the whey proteins associated with the casein micelle. Skimmed milk with added whey protein aggregates (60 nm) showed a very similar behaviour to skimmed milk where only native whey proteins were added, indicating a negligible effect of the whey protein aggregates on the rate of flocculation. These results are in agreement with Steffl (26), who demonstrated that protein particles smaller than 100 nm do not affect the gel strength in any way. Also the aggregates formed in milk during heat treatment are smaller than 100 nm. The only other factor left is the whey protein coating of the casein micelles, which seems a likely candidate, as at pH 6.7 para-κ-casein itself is uncharged while a para-κ-casein whey protein complex retains a charge. The pH at which heat treatment is performed increases the rate of flocculation in the following order 6.55, 6.7, 6.45, 6.9, 6.35 and unheated milk. The schematic representation (Figure 4.4) indicated that at pH 6.55 most whey proteins are present on the casein micelle with a rather homogenous distribution. Milk heated at pH 6.7 and 6.9 seems to have a similar distribution of whey proteins, but due to the formation of whey protein aggregates fewer whey protein molecules are involved in interactions with κ-casein, which probably causes the slightly improved renneting behaviour. At pH 6.55 to 6.35 all whey proteins are present on the micelle, but with lower pH larger structures are present on the casein micelle, leaving many κ-casein molecules unreacted. The results further confirm the model presented in Figure 4.4. Adjusting the pH prior to heat treatment is a promising tool to improve the rennet-induced clotting of heated milk. It might allow inclusion of whey proteins in the cheese curd while retaining clotting properties comparable to unheated milk

Acid-induced gelation of heated milk

Figure 4.6 shows the $\tau_{1/2}$ as a function of pH of the milks heated at pH 6.35 to 6.9 as determined by DWS. In the pH range of 6.9 to 6.45 a steady decrease in gelation pH is observed, while milk heated at pH 6.35 has a similar gelation behaviour as milk heated at pH 6.55.

In all milks the total degree of denaturation is rather similar, while the gelation pH differs, showing that the total degree of denaturation is clearly not determining the gelation pH. Apparently, the distribution of whey proteins in aggregates and coating of the casein micelles affects the gelation pH. Schorsch et al. (22) demonstrated that addition of whey protein aggregates to micellar casein causes a pH of gelation which is even higher than that seen when whey proteins and micellar casein are heated together (gelation pH of 6.0 versus 5.3). This demonstrates that aggregates have a large effect on the gelation pH. Therefore, the whey protein aggregates formed during heat treatment at both pH 6.9 and 6.7 can explain the high gelation pH. At pH 6.9 even more whey protein aggregates are formed than at pH 6.7, resulting in a higher gelation pH than that of milk heated at pH 6.7. When milk is heated at pH 6.55 to 6.35 all whey proteins are associated with the casein micelle. The absence of whey protein aggregates in milk heated at pH 6.55 explains the decrease in gelation pH as compared to milk heated at pH 6.7. The

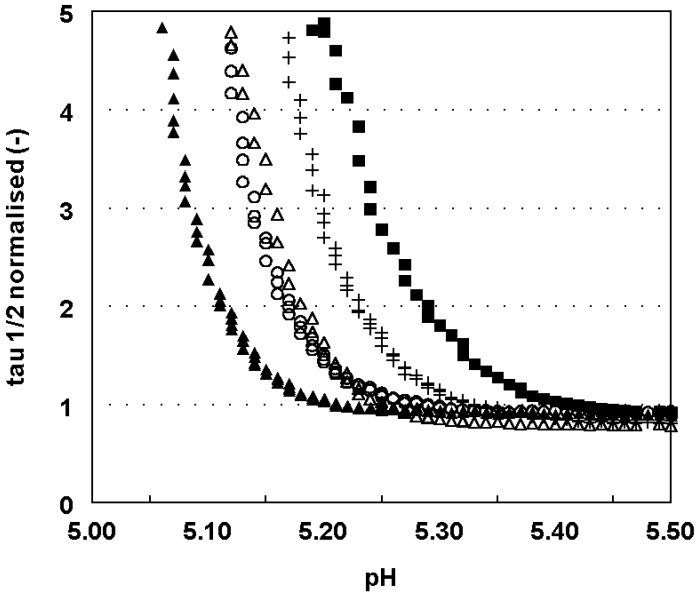

Figure 4.6: $\tau_{1/2}$ as function of pH obtained by DWS of milk heated for 10 min at 80°C at a pH of 6.9 (■), 6.7 (+), 6.55 (○), 6.45 (▲) and 6.35 (△) and acidified with 1.2% GDL at 32°C.

further decrease in gelation pH of milk heated at pH 6.45 can only be explained by the type of the whey protein coating as all other factors are identical to milk heated at pH 6.55. The previous results on the type of whey protein coating of the casein micelle (Figure 4.3) showed that heat treatment at lower pH causes a more inhomogeneous coating. Probably this causes a lower gelation pH due to the larger patches of κ-casein molecules without associated whey proteins, leading to a gelation behaviour closer to unheated milk. At pH 6.35 an even lower gelation pH would be expected due to this phenomenon. However, an increase in gelation pH is observed which seems likely to be caused by the formation of rather large whey protein aggregates on the surface of the micelle. These associated whey protein aggregates might affect the gelation pH in a similar way as soluble whey protein aggregates do. Controlling the pH of milk prior to heat treatment appears to be very important in order to obtain a constant end product. Additionally, varying the pH prior to heat treatment allows modification of the final product by using the same starting material.

Conclusions

Summarising, it is concluded that heat treatment of milk at pH values of 6.35 to 6.9 has large effects on rennet- and acid-induced gelation. These effects can be attributed to the interaction of denatured whey proteins with casein micelles and with each other during heat treatment of milk. A schematic representation of the interactions between casein micelles and whey proteins as function of pH of heat treatment is shown in Figure 4.4. This model enables us to interprete all of our experimental observations in a consistent manner. Soluble aggregates have a large effect on the acid-induced gelation pH, while they hardly affect renneting. The amount of whey proteins associated with the casein micelle, and even more so the type of whey protein coating on the casein micelle, has large effects on both the rennet- and the acid-induced gelation. This chapter shows the relevance of slight pH changes on the quality of milk derived products. For example the slight changes in pH of milk which can occur during the year can have large effects on cheese and yoghurt production and should be taken into consideration if a constant quality of the end product is desired. Additionally, this knowledge might provide opportunities to design new products such as cheese

containing denatured whey proteins but with similar curd formation characteristics as unheated milk.

References

1. Alting, A.C., R.J. Hamer, C.G. de Kruif and R.W. Visschers. 2000. Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. J. Agric. Food Chem. 48: 5001.
2. Anema, S.G. and H. Klostermeyer. 1997. Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100°C. J. Agric. Food Chem. 45: 1108.
3. Burova T.V., N.V. Grinberg, R.W. Visschers, V.Ya. Grinberg and C.G. de Kruif. Calorimetric study of porcine β-lactoglobulin at different pH. submitted for publication in Biochim. Biophys. Acta.
4. Corredig, M. and D.G. Dalgleish. 1996. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Res. Int. 29: 1: 49.
5. Dannenberg, F. and H.G. Kessler. 1988. Reaction kinetics of the denaturation of whey proteins in milk. J. Food Sci.53: 258.
6. Hill, A.R. 1989. The β-lactoglobulin-κ-casein complex. Can. Inst. Food Sci. Technol. J. 22: 2: 120.
7. Hoedemaeker, F.J., R.W. Visschers, A.C. Alting, C.G. de Kruif, M.E. Kuil, J.P. Abrahams. 2002. Structure of β-lactoglobulin from pig (Sus scrofa): A novel dimer interface. Acta Cryst. D. 58 : 480.
8. Hoffmann, M.A.M.. β-lactoglobulin: denaturation and aggregation. Ph.D. Diss., University of Utrecht, Utrecht, Neth.
9. Jang, H.D. and H.E. Swaisgood. 1990. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. J. Dairy Sci. 73: 900.
10. de Kruif, C.G., M.A.M. Hoffmann, M.E. van Marle, P.J.J.M. van Mil, S.P.F.M. Roefs, M. Verheul and P. Zoon. 1995. Gelation of proteins from milk. Faraday Discuss. 101: 185.
11. Law, A.J.R., D.S. Horne, J.M. Banks and J. Leaver. 1994. Heat-induced changes in the whey proteins and caseins. Milchwissenschaft. 49: 3: 125.
12. Law, A.J.R. and J. Leaver. 2000. Effect of pH on the thermal denaturation of whey proteins in milk. J. Agric. Food Chem. 48: 672.
13. Lucey, J.A., C. Gorry, B. O'Kennedy, M. Kalab, R. Tan-Kinita and P.F. Fox. 1996. Effect of acidification and neutralization of milk on some physico-chemical properties of casein micelles. Int. Dairy J. 6: 257.
14. Noh, B. and T. Richardson. 1989. Incorporation of radiolabelled whey proteins into casein micelles by heat processing. J. Dairy Sci. 72: 1724.
15. Noh, B., T. Richardson and L.K. Creamer. 1989. Radiolabelling study of the heat-induced interactions between α-lactalbumin, β-lactoglobulin and κ-casein in milk and in buffer solutions. J. Food Sci. 54: 4: 889.

16. Oldfield, D.J., H. Singh and M.W. Taylor. 1998. Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. Int. Dairy J. 8: 765.
17. Oldfield, D.J., H. Singh, M.W. Taylor, K.N. Pearce. 2000. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk, Int. Dairy J. 10: 509.
18. Paulsson, M., Thermal denaturation and gelation of whey proteins and their adsorption at the air/water interface. Ph.D. Diss., University of Lund, Lund, Sweden.
19. Rasmussen, L.K., Højrup, P. & Petersen, T.E. (1994). Disulfide arrangement in bovine caseins: lacalization of intrachain disulfide bridges in monomers of κ- and α_{s2}-casein from bovine milk. Journal of Dairy Research, 61, 485-493.
20. Rasmussen, L.K. and T.E. Petersen. 1991. Purification of disulfide-linked α_{s2}- and κ-casein from bovine milk. J. Dairy Res. 58: 187.
21. Roefs, S.P.F.M., C.G. de Kruif. 1994. A model for the denaturation and aggregation of β-lactoglobulin. Eur. J. Biochem. 226: 883.
22. Schorsch, C., D. K. Wilkins, M.G. Jones and I.T. Norton. 2001. Gelation of casein-whey mixtures: effects of heating whey proteins alone or in the presence of casein micelles. J. Dairy Res. 68: 471.
23. Singh, H.S. 1993. Heat-induced interactions of proteins in milk. Protein and fat globule modifications-IDF seminar, special issue 9303. 191.
24. Singh, H., S.I. Shalabi, P.F. Fox, A. Flynn and A. Barry. 1988. Rennet coagulation of heated milk: influence of pH adjustment before or after heating. J. Dairy Res. 55: 205.
25. Singh, H. and A. Wauguna. 2001. Influence of heat treatment of milk on cheese making properties. Int. Dairy J. 11: 543.
26. Steffl, A., R. Schreiber, M. Hafenmaier and H. Kessler. 1999. Influence of whey proteins aggregates on the renneting properties of milk. Int. Dairy J. 9: 403.
27. Ugolini R., L. Ragona, E. Siletti, F. Fogolari, R.W. Visschers, A.C. Alting and H. Molinari. Aggregation, Stability and Electrostatic potential properties of porcine β-lactoglobulin. Eur. J. Biochem. 268: 4477.
28. Vasbinder, A.J., A.C. Alting and C.G. de Kruif. Manuscript in preparation. Heat-induced casein-whey protein interactions in milk. Chapter 3 of this thesis.
29. Vasbinder, A.J., A.C. Alting, R.W. Visschers and C.G. de Kruif, Texture of acid milk gels: formation of disulfide cross-links during acidification. Submitted for publication in the Int. Dairy J. Chapter 7 of this thesis.
30. Vasbinder, A.J., P.J.J.M. van Mil, A. Bot and C.G. de Kruif. 2001. Acid-induced gelation of heat treated milk studied by Diffusing Wave Spectroscopy. Colloids and Surfaces B. 21: 245. Chapter 6 of this thesis
31. Walstra, P.and R. Jenness. 1984. Dairy Chemistry and Physics, John Wiley and sons, Inc, USA.

Chapter 5: Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics

Abstract

Casein micelles in milk are stable colloidal particles stabilised by a hairy brush of κ-casein. During cheese production rennet cleaves κ-casein into casein macropeptide and para-κ-casein, thereby destabilising the casein micelle and resulting in aggregation and gel formation of the micelles. Heat treatment of milk causes impaired clotting properties which makes heated milk unsuitable for cheese production.

In this paper we compared five different techniques, often used in the literature, for their suitability to quantify the enzymatic hydrolysis of κ-casein. It was found that the technique applied is crucial for the yield of casein macropeptide and this yield then affects the calculated enzymatic inhibition caused by heat treatment, ranging from 5 to 30%. Using the technique, which was validated to be the most reliable, demonstrates that heat-induced calcium phosphate precipitation does not affect the enzymatic cleavage, while whey protein denaturation causes a very slight reduction of enzyme activity. By using diffusing wave spectroscopy, a very sensitive technique to monitor gelation processes, we demonstrated that heat-induced calcium phosphate precipitation does not affect the clotting. Whey protein denaturation does not affect the start of flocculation but has a clear effect on the clotting process. This work adds to a better understanding of the processes causing the impaired clotting properties of heated milk.

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Introduction

A casein micelle is an association colloid with a stabilising hairy brush of calcium-insensitive κ-casein on its surface. Chymosin, the major enzyme in rennet, is an endopeptidase which in milk cleaves very specifically the Phe105-Met106 bond of κ-casein. The subsequently formed para-κ-casein is insoluble, while the released casein macropeptide (CMP) is soluble. Para-κ-casein lacks the colloid-protective property of κ-casein; therefore, extensive cleavage of the κ-caseins will result in destabilisation and aggregation of the micelles into a coagulum (4, 22). Manufacturing of cheese is preferably carried out with unheated or low-pasteurised milk. Under these conditions the action of the chymosin is well investigated. The activity of the enzyme is dependent on pH, temperature and calcium concentration. An overview of the effects of these parameters on enzymatic cleavage, clotting and syneresis is provided by Walstra and Jenness (22).

Chymosin acts only on the casein micelles; native whey proteins are not included in the curd. Upon heat treatment whey proteins interact with the micelles through thiol group-disulfide bridge exchange reactions (8) and are transferred to the curd. However, heat treatment of the milk leads to off flavours in cheese and causes impaired clotting properties resulting in a weaker curd and therefore making it less suitable for cheese manufacturing (17, 23). In the literature various explanations are given for this phenomenon, which either alone or in combination would cause the impaired clotting, such as 1] incomplete enzymatic hydrolysis, 2] reduced concentration of serum calcium due to precipitation of calcium phosphate and 3] stabilisation of casein micelles due to the coating with denatured whey proteins.

1] Incomplete enzymatic hydrolysis

Most papers on renneting of heated milk indicate that the interaction of β-lactoglobulin with κ-casein causes incomplete enzymatic hydrolysis resulting in “fairly” stable casein micelles (5, 14, 16, 22, 24, 25). However, there are papers where this is disputed (7, 10). These conflicting results can partly be explained by the method used for the CMP determination. In most studies (5, 7, 14, 16, 24, 25) the quantification was based on fractionation by trichloro-acetic acid

(TCA) which causes part or all of the non-glycosylated CMP (40% of total CMP) to precipitate at concentrations above 2%. At high TCA concentrations even glycosylated CMP starts to precipitate depending on the degree of glycosylation (20). Therefore, in studies based on TCA fractionation using concentrations of 2 to 12%, only a limited part of CMP is taken into account, not necessarily representative of the complete fraction of CMP. In the present paper we compared five different techniques to monitor the enzymatic activity of rennet in heat treated milk, i.e. precipitation by acetic acid and by TCA (2, 8 and 12%) followed by CMP analysis, and monitoring of para-κ-casein. All isolation methods were combined with RP-HPLC, which is a very suitable technique for quantification of CMP and para-κ-casein (12, 20).

2] Decrease in serum calcium due to calcium phosphate precipitation

Upon heat treatment of milk calcium phosphate precipitates, causing a decrease in the concentration of serum calcium. The lower calcium concentration might prevent aggregation of renneted casein micelles (6). This conclusion is based on the positive effects that addition of extra calcium has on the renneting properties of heated milk and the improved gel strength obtained by pH cycling of heated milk prior to renneting (6, 16, 17). However, none of these studies gives direct evidence for the relation between precipitated calcium and the impaired clotting of heated milk. Schreiber (15) showed that UHT treatment (100-140°C) of whey-protein-free milk (WPF-milk) resulted in impaired gel-forming abilities. However, such experiments have not, to our knowledge, been done at the temperature regime of 70-90°C. In this paper we investigated the effects of heat treatment on WPF-milk at temperatures ranging from 70 to 90°C.

3] Stabilisation of casein micelles due to interaction with denatured whey protein

At pH 6.7 whey proteins, precipitated at the surface of the micelle, are positively charged and stabilise the micelle even when the CMP is released. Dalgleish (4) found a correlation between the amount of denaturated whey proteins and the rennet clotting time irrespective of the temperature of heat treatment. Other authors observed similar trends (13, 17, 18, 23). In general an increased

degree of whey protein denaturation results in an increased rennet clotting time (RCT) and a decreased gel strength. However, in these studies the two above mentioned effects (1 and 2) were not taken into account and no direct proof of an effect of whey protein denaturation on these properties was given. In this paper we compared the effect of heat treatment of WPF-milk and of WPF-milk with added whey proteins on the clotting properties as monitored by DWS. In this case whey protein addition is the only change made to the system. This allows us to investigate the effect of calcium phosphate precipitation on the clotting properties and the additional contribution of whey protein denaturation.

Materials and Methods

Skim milk

Skim milk heated for 10 seconds at 72°C was obtained from the pilot plant at NIZO food research, Ede. Sodium azide (0.02%) was added to prevent bacterial growth and the milk was stored at 4°C.

Whey Protein Isolate (WPI) solution

Whey Protein Isolate (Bipro; Davisco Food International Inc., USA; for composition see materials and methods chapter 3) solution was prepared at a concentration of 9% (w/w). The solution was stirred for two hours at room temperature and subsequently filtered though a 0.45μm filter; 0.02% (w/w) NaN_3 was added.

Whey-protein-free milk

Reconstituted WPF-milk was prepared by dissolving 9.23 g of milk powder prepared by microfiltration and ultrafiltration (NIZO food research, Ede) in 90.77 g of distilled water. The composition of this milk is discussed in the materials and methods section of chapter 2. The milk was stirred at 45°C for 1 hour. To prevent bacterial growth 0.02% NaN_3 was added and the milk was kept overnight at 4°C before use. The initial pH of the milk was 6.67 (± 0.01). Before further use 19.45 g of WPF-milk was mixed with either 1 g of distilled water or WPI solution, resulting in a final concentration of 8.78% (w/w).

Heat treatment of milk

The cold milk was adjusted to room temperature for 2 hours. Glass tubes (8 ml, diameter 1 cm) were filled with 5 ml of milk and heated for 10 min in a water-bath at the required temperature ranging from 70 to 90°C and cooled with tap water to room temperature.

Renneting of the milk for CMP and para-κ-casein analysis

The heat-treated milk was incubated for 75 min at 30°C. An aliquot was withdrawn (blank) before addition of rennet. Then 500 times diluted rennet (CSK 10.800, the Netherlands, 150 IMCU/ml) was added to a final concentration of 0.005% rennet in the milk. After 4 hours of incubation the enzymatic reaction was stopped by addition of 10µl/ml of pepstatin solution (5mg pepstatin (Sigma) / ml DMSO). These milk samples were used for CMP and para-κ-casein isolation.

CMP isolation

Acetic acid precipitation

An amount of 0.4 g of renneted milk was mixed with 0.8 g of distilled water (40°C) and 40 µl acetic acid (HAc) (10%) in an Eppendorf tube (2 ml). After mixing (vortex) and 10 min waiting, 40 µl of sodium acetate (NaAc; 1M) and 0.72 g of distilled water were added and the solution was mixed again. After 1 hour standing the solution was centrifuged for 5 min at 3000g. The CMP in the supernatant was determined by RP-HPLC.

TCA precipitation

The renneted milk was mixed in an Eppendorf tube with a 24% (w/w) TCA solution to a final concentration of 2, 8 and 12% TCA (w/w), stirred thoroughly and incubated for 30 min at 20°C. The sample was then centrifuged for 5 min at 5000g and the supernatant was mixed with buffer (0.1M bis-tris pH7, 8M urea, 45mM citrate) to a final pH of 3.0. The supernatant : buffer ratios were 1:1 for 2% TCA and 1:2 for 8 and 12% TCA. The mixture was filtered though a 0.22µm filter before injection on the RP-HPLC column.

Para-κ-casein isolation

Renneted milk was mixed 1:1 with buffer (0.1M bis-tris pH7, 8M urea, 45mM citrate) containing mercaptoethanol (0.3% v/v). After 1 hour of incubation at 20°C, 0.5g of this solution was added to 1.5g of buffer (6M urea, adjusted to pH 3 with TFA). The sample was mixed thoroughly, filtered through a 0.22 μm filter and analysed by RP-HPLC.

RP-HPLC analysis of CMP

A description of the RP-HPLC method used is given by Minckiewicz et al. (12). A gradient was applied of solvent A (acetonitrile:water:trifluoroacetic acid (TFA) 100:900:1, v/v/v) and solvent B (same components 900:100:0.8, v/v/v). The gradient was started with 15% of solvent B and increased from 15-28% over 13 min, 28-32% over 22 min; 32-70% over 3 min and finally kept constant for 5 min before returning to the initial conditions. Analytical separations were carried out at 30°C, a flow rate of 0.8 ml/min, peak detection at 220 nm and an injection volume of 100μl (Hi-Pore reversed phase C18 column).

RP-HPLC analysis of para-κ-casein

Apart from a different gradient applied and an injection volume of 50 μl the same protocol as presented in HPLC analysis of CMP was used for para-κ-casein. The gradient was started with 26% of solvent B and increased from 26-40% over 50 min, 40-70% over 2 min and finally kept constant for 5 min before returning to the initial conditions.

DWS measurement of renneted milk

The heat-treated milk was incubated for 75 min at 30°C. Then 500 times diluted rennet was added to a final concentration of 0.005% in the milk. The rennet-induced clotting of the milk was monitored by DWS. Light from a 5 mW He-Ne laser (632.8 nm) was passed through a multi-mode fibre into the milk. The back-scattered light was monitored by a single-mode fibre located at 3.0 mm from the input fibre. The scattered light was detected with a photo multiplier tube (ALV SO-SIPD and fed to a PC-interfaced autocorrelator (Flex 5000, correlator.com). The correlator calculates the autocorrelation

functions in real time. The time at which the autocorrelation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{1/2}$ (see Figure 6.2) and the value is determined by linear interpolation. The renneting was monitored in time at intervals of 2 min. All data were normalised by the τ ½ value (average of 5 measurement of 1 min) of the same sample prior to rennet addition. This eliminates variations between the samples and fibers. The gelation point is defined in the plot of $\tau_{1/2}$ against renneting time as the time at which $\tau_{1/2}$ is increasing. The error in determining the gelation point is at most 5 minutes.

Results and Discussion

Rennet-induced gelation of heated skim milk studied by DWS

The rennet-induced clotting of casein micelles in pre-heated skim milk monitored by DWS, as a function of time, is shown in Figure 5.1 . The parameter $\tau_{1/2}$ reflects the mobility of the particles in solution, i.e. the casein micelles in milk, and is therefore a sensitive parameter for monitoring the clotting of the micelles and the beginning of the subsequent gelation. All curves show a gradual and reproducible decrease in $\tau_{1/2}$ towards a minimum at 200 min. This minimum is followed by an increase in $\tau_{1/2}$, but lower values are reached with increasing temperature of heat treatment (70 to 90°C). Unheated milk and milk heated at 70°C show a rather similar

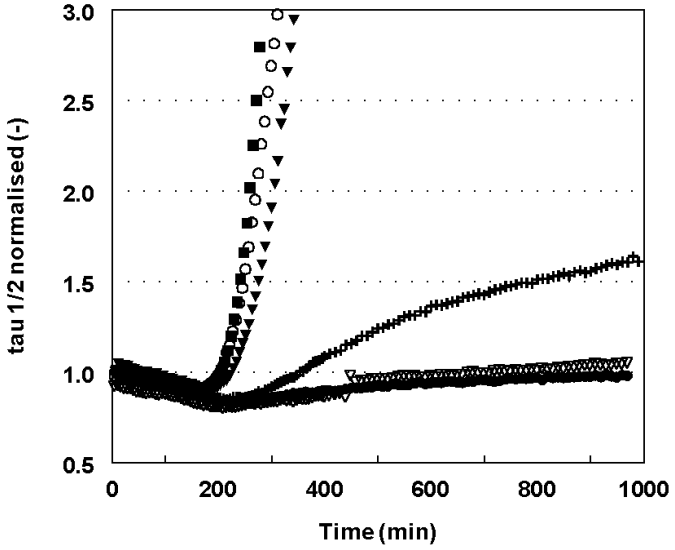

Figure 5.1: The $\tau_{1/2}$ traces of fresh skim milk unheated (○) and heated at at 70 (■), 75 (▼), 80 (✚), 85 (●) and 90°C (▽) for 10 min subjected to rennet treatment at 30°C.

behaviour, a steep increase in $\tau_{½}$ during renneting. The $\tau_{½}$ curves of milk heated at 85 and 90°C are both hardly increasing.

The decrease of $\tau_{½}$ to a minimum in the first 200 min of incubation means that the mobility of the micelles increases during renneting due to a decrease in size which is caused by enzymatic cleavage of the κ-caseins in the hairy brush (9, 11, 21). The fact that this mimimum is observed for all the milks studied demonstrates that proteolytic cleavage takes place both in unheated and heated milk. All studies published on RCT's of heat-treated milk report that increasing the temperature of heat treatment results in an increase in RCT. The RCT is defined as the time at which flocculation is observed visually. For heated milks it is increased by a factor 10-15 relative to unheated milk (1, 4, 16). DWS, which is a very sensitive technique for measuring flocculation, adds complementary information to these observations. It demonstrates that the onset of clotting of milk is not affected by the heat treatment; but only the kinetics of flocculation. This means that the micelles aggregate independent of the heat treatment applied, but the casein micelles are no longer able to form a gel. This latter observation corresponds with the increased RCT and gelation times and the decreased G' values as reported in the literature (1, 4, 6, 13, 16, 18, 23), which is ascribed to a decrease in enzymatic activity, precipitation of calcium and denaturation of whey proteins. In this paper we systematically investigate the effect of heat treatment on these three parameters by comparing fresh skim milk, WPF-milk and WPF-milk with added whey proteins.

Enzymatic hydrolysis in heated skim milk

To study the enzymatic activity in heated milk various methods are possible, i.e. CMP isolation by precipitating the other milk proteins with acetic acid / sodium acetate precipitation (Hac/NaAc) or with 2, 8 and 12% TCA, or determination of para-κ-casein. In this section we will compare the four different CMP isolation techniques with respect to quantity and composition of CMP present in the supernatant. All five techniques were used to determine the enzymatic activity in milk heated at 90°C for 10 min. The technique which was validated to be the most reliable was used to study the enzymatic hydrolysis in heated milk.

Comparison of four CMP isolation methods in unheated milk

Figure 5.2 shows four RP-HPLC chromatograms of CMP present in unheated milk treated with rennet and isolated by precipitation with HAc/NaAc and with 2, 8 and 12% TCA. The figure reveals that the isolation technique used has a large effect on the total area of the peaks present in the chromatogram. The shape and position of the RP-HPLC pattern of supernatants obtained by HAc/NaAc and 2% TCA precipitation are almost identical. However, with increasing TCA concentration a clear decrease in area is observed. The non-glycosylated CMP B peak disappears completely after precipitation with 8 and 12% TCA. Non-glycosylated CMP A precipitated partly in 8% and almost completely in 12% TCA. Table 5.1 shows the total area of CMP, the area of glycosylated and non-glycosylated CMP, and non-glycosylated CMP as a percentage of total

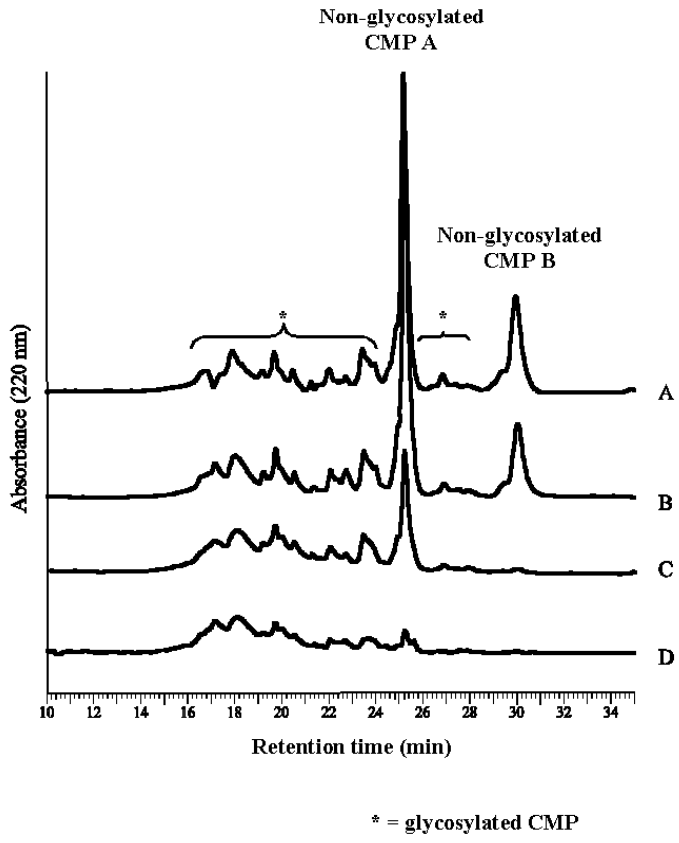

Figure 5.2: RP-HPLC chromatograms of CMP released by rennet treatment of unheated fresh skim milk. Isolation of CMP occurred by precipitation of the proteins in milk with acetic acid / sodium acetate (A), 2% TCA (B), 8% TCA (C) and 12% TCA (D). Indicated in the figure are the peaks corresponding with non-glycosylated CMP A and B and the glycosylated CMP's.

CMP isolation method	total CMP (V*s)	glycosylated CMP (V*s)	non-glycosylated CMP (V*s)	% non-glycosylated CMP of total CMP
HAc/NaAc	35±1 [a]	16±2 [a]	20±1 [a]	55±3 [a]
2% TCA	48±4 [b]	25±2 [b]	23±3 [a]	48±3 [a]
8% TCA	16±3 [c]	13±3 [a]	3±1 [b]	21±1 [b]
12% TCA	11±1 [c]	11±1 [a]	0±0 [b]	2±1 [c]

Table 5.1: peak area of CMP, determined by RP-HPLC, in renneted unheated milk (4 hours, 0.005% at 30°C) isolated by precipitation with acetic acid / sodium acetate (HAc/NaAc), 2, 8 and 12% TCA. The CMP is presented as total CMP, glycosylated CMP and non-glycosylated CMP. The non-glycosylated CMP is composed of the peaks of CMP A and B as indicated in Figure 5.2. In the last column the percentage of non-glycosylated CMP obtained is shown. The results are an average of 4 independent measurements. The standard error of the mean is indicated in the table. The superscript letters a, b and c are based on an analysis of the variance among the methods, showing if a difference in yield and percentage between the 4 methods is significant.

CMP. With increasing TCA concentration the area of total CMP diminishes by 60 and 80%, for 8 and 12% TCA respectively, compared to 2% TCA. The same trend is observed for the non-glycosylated CMPs, but the decrease is even stronger, i.e. about 90and 100% for 8 and 12% TCA respectively. The area of glycosylated CMPs shows a clear decrease for 8 and 12% TCA, compared to 2% TCA. Isolation by acetic acid precipitation yields less total CMP, glycosylated and non-glycosylated CMP than with 2% TCA. Although the absolute yield is clearly less than by isolation with 2% CMP the relative amounts are very similar. Non-glycosylated CMP represents about 50% of the total in the case of HAc/NaAc and 2% TCA, but only 21 and 2% for 8 and 12% TCA, respectively. This is in agreement with the results of Vreeman et al. (20), who showed that 90% of the non-glycosylated CMP precipitates at 8% TCA and 100% precipitates at 12% TCA. They also observed that at very high TCA concentrations (12%) even glycosylated CMP becomes sensitive to precipitation. We can conclude that the technique used for CMP isolation is crucial for the yield of CMP observed in the supernatant. For determination of the absolute amounts of CMP present in milk, isolation with 2% TCA seems favourable, for relative measurements both techniques seem reliable.

Isolation with 8 and 12% TCA yields only a fraction of total CMP and non-glycosylated CMP is precipitated selectively.

Comparison of five methods to study enzymatic hydrolysis in heated milk

The effect of heat treatment of milk (10 min at 90°C) on the enzymatic activity of rennet monitored by the four different CMP isolation methods and determination of para-κ-casein is depicted in Figure 5.3. The amount of CMP and para-κ-casein released was determined after 4 hours of incubation, which is the time at which a visible gel was formed in unheated milk and milk heated at 70°C. At this point 85% of CMP was released in unheated milk. For all fractionation methods used the amount of CMP released is expressed as the percentage of the area observed for the unheated milk. At 90°C the percentage of CMP released is 95, 92, 67, 78 and 88% in the case of precipitation by HAc/NaAc, 2, 8 and 12% TCA and determination of para-κ-casein, respectively. The standard deviations are indicated in the figure. Precipitation by 2% TCA clearly shows the lowest standard deviation.

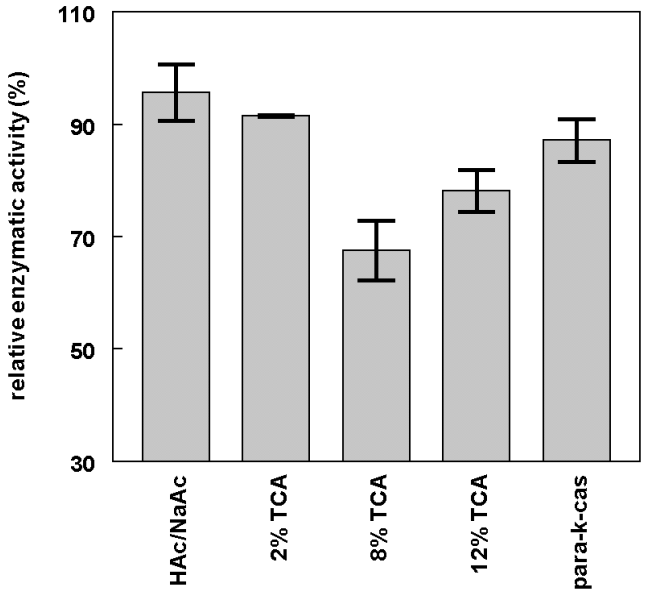

Figure 5.3: Relative enzymatic hydrolysis (%) in fresh skim milk heated for 10 min at 90°C. Comparison of five different methods to determine hydrolysis, i.e. precipitation of the proteins in milk by acetic acid / sodium acetate (Hac/NaAC) and 2, 8 and 12% TCA, and monitoring of para-κ-casein. The degree of hydrolysis is represented as percentage of hydrolysis determined in unheated milk with the same isolation method. In the graph the standard deviation of the different methods is indicated.

These results reveal that analysis of the same samples with different isolation techniques can cause almost 30% difference in determined degree of hydrolysis. This may lead to completely different conclusions. The results obtained with HAc/NaAc and 2% TCA show only a limited inhibition of the enzymatic activity induced by heat treatment, while the results with 8% TCA suggest a significant inhibition of the enzymatic activity. Comparing these findings with reports in the literature on enzymatic cleavage of κ-casein shows that in several studies fractionation was carried out with 2% and 12% TCA and analysed by determining the total nitrogen content (5, 24, 25). In 2% TCA both carbohydrate-containing and carbohydrate-free macropeptides are soluble; however, their quantification by determination of total nitrogen is difficult due to large amounts of nitrogenous material, like native whey proteins, which is also soluble in the milk (2). Therefore, the comparison of unheated and heated milks by this procedure is hampered as denatured whey proteins are precipitated in 2% TCA. This explains the large decrease in soluble nitrogen found in 2% TCA for heated milk compared to unheated. Combining 2% TCA precipitation with RP-HPLC, which separates the whey proteins from CMP, yields a very reliable technique. Experiments performed by precipitation with 8% TCA show variations in CMP release ranging from 10% to 30% (14, 7). In contrast, Marshall (10) showed that determination of the enzymatic activity by monitoring the release of para-κ-casein in milk heated for 10 min at 80°C caused only 4% reduction of enzymatic hydrolysis compared to unheated milk. Apart from this paper most papers show a clear decrease in enzymatic cleavage, which is also the general opinion held in the literature (3, 22). Here we conclude that enzymatic activity or, better, the amount of CMP split off is only slightly influenced by heating milk at 90°C (10 min). The variable results in the literature reflect insufficient awareness of the interference of the analytical methods with the interpretetion of experimental data (table 5.1; 20).

Enzymatic hydrolysis as a function of temperature of heat treatment

In Figure 5.4 the enzymatic hydrolysis which is reached after 4 hours of renneting of fresh skim milk and WPF-milk is shown as a function of the temperature of heat treatment. The CMP isolation was performed by precipitation with 2% TCA as this results in a maximum yield of CMP, both glycosylated and non-glycosylated, and the results show a very low standard deviation. Heat treatment at

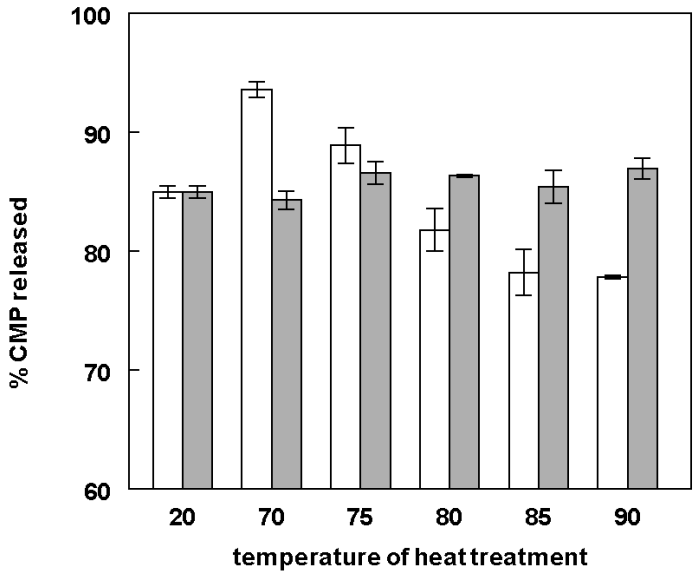

Figure 5.4: Enzymatic hydrolysis of fresh skim milk (white bars) and WPF-milk (grey bars) subjected to heat treatment ranging from 70 to 90°C represented by % CMP released which was isolated by precipitation with 2% TCA. The standard deviation is indicated in the graph.

70°C causes a slight increase in enzymatic hydrolysis compared to unheated milk which slowly levels off at higher temperatures of heat treatment to 92% of unheated milk. No effect of heat treatment was observed for WPF-milk: at all temperatures of heat treatment the release was similar to the unheated WPF-milk.

Comparing the results of fresh skim milk and WPF-milk shows two differences, i.e. the maximum in CMP release at 70°C for fresh skim milk and the slight reduction in CMP release at 85 and 90°C. The absence of whey proteins in WPF-milk allows us to study the heat-induced calcium phosphate precipitation. The results demonstrate that heat treatment has no effect on enzymatic hydrolysis; therefore we can exclude heat-induced calcium phosphate precipitation as a factor influencing the enzymatic hydrolysis. The maximum in CMP release observed in milk heated at 70°C has not previously been reported in the literature. A restructuring of the casein micelle due to mild heat treatments could increase the accessibility of the κ-caseins. In this case WPF-milk is probably not a good model system as it is spray-dried and reconstituted. Although, more research is required in order to understand the mechanism causing this maximum; it can be concluded that it does not affect the rate of clotting (Figure 5.1). As heat-induced calcium phosphate precipitation has no effects on enzymatic hydrolysis, it seems very likely that the small reduction in enzymatic hydrolysis at higher

temperatures of heat treatment is caused by the denaturation of whey proteins, which is the general opinion in the literature (3, 22). However, the reduction is very limited, i.e. 6% less than in unheated milk, while heat treatment at 70°C shows an increase of 9%. Comparing unheated milk and milk heated for 10 min at 80°C (60% of denaturation) shows an enzymatic activity which is very similar for both milks, while the flocculation rate decreased by a factor of 2 (Figure 5.1). These observations demonstrate clearly that slight changes in enzymatic hydrolysis cannot explain the observed effects on the rate of clotting.

Rennet-induced gelation of heated milk

Calcium phosphate precipitation

Figure 5.5 illustrates the effect of heat treatment on WPF-milk as monitored by DWS. A steady decrease of $\tau_{½}$ towards a minimum was observed, which is situated around 150 min, followed by a sharp increase. The milks behaved identically under all the heat treatments applied. The start of flocculation of WPF-milk occurred 50 min earlier than in the case of fresh skim milk. Apparently, spray drying and reconstitution of the milk affect the stability of the casein micelles. Heat treatment of WPF-milk did not affect the gelation properties. The major difference between skim milk and WPF-milk is the absence of whey proteins; therefore, comparison of the two milk

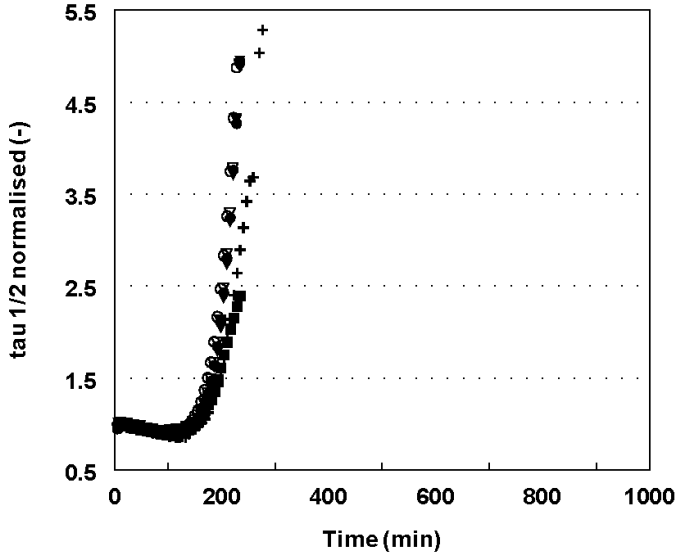

Figure 5.5: The $t_{½}$ traces of WPF-milk unheated (○) and heated for 10 min at 70 (■), 75 (▼), 80 (✚), 85 (●) and 90°C (▽) subjected to rennet treatment at 30°C.

types should reveal the effect of heat-induced calcium precipitation, which is usually masked by the effect of whey protein denaturation. Raynal and Remeuf (13) observed a 10-15% loss of diffusible calcium in skim milk upon heat treatment (10 min, 75-90°C). However, heat treatment did not affect the clotting of WPF-milk, which indicates that 10-15% loss of calcium does not affect this process. At temperatures above 100°C effects of heat treatment on renneting properties of WPF-milk were found (15). A clear decrease in the gel strength was observed. The higher the temperatures applied (100-140°C) the weaker the final rennet gels obtained (60 to 90% weaker than unheated milk). A decrease in soluble calcium of 30 and 50% was caused by a heat treatment for 10 min at 100 and 120°C, respectively (15). This indicates that precipitation of a significant amount of calcium can affect the gel formation. By interpolating these results to the temperature regime of 70-90°C it seems likely that a reduction in gel strength would occur. However, this has not yet been investigated. The role of calcium with respect to rennet-induced gelation appears to be rather complicated. At temperatures of heat treatment of 70 to 90°C it has no effect on the clotting of the casein micelles but it seems likely to influence the gel strength. However, one main conclusion can be drawn: at heat treatments below 100°C precipitation of calcium is not responsible for the impaired clotting of heated skim milk.

Whey protein denaturation

Figure 5.6 shows the effect of whey protein denaturation caused by heat treatment on the rennet-induced gelation of WPF-milk with added whey proteins as measured with DWS. All curves show a slight decreases in $\tau_{½}$ towards a minimum around 150 min, followed by an increase which is dependent on the heat treatment applied. Unheated milk and milk heated at 70°C show an almost identical behaviour; heat treatment at 75 and 80°C caused a less steep increase of the DWS trace but still a steady increase was observed. Milk heated at 85 and 90°C showed a weaker increase of $\tau_{½}$ which levelled off at 600 min. No gel formation was observed in these milks. Comparing Figures 5.5 and 5.6 shows that addition of whey proteins causes changes in the flocculation behaviour of the casein micelles. The minimum is not affected by the heat treatment applied both in either the presence or the absence of whey proteins. This reveals that casein micelles start to interact and to form flocs independent of the presence of whey proteins. In the presence of whey

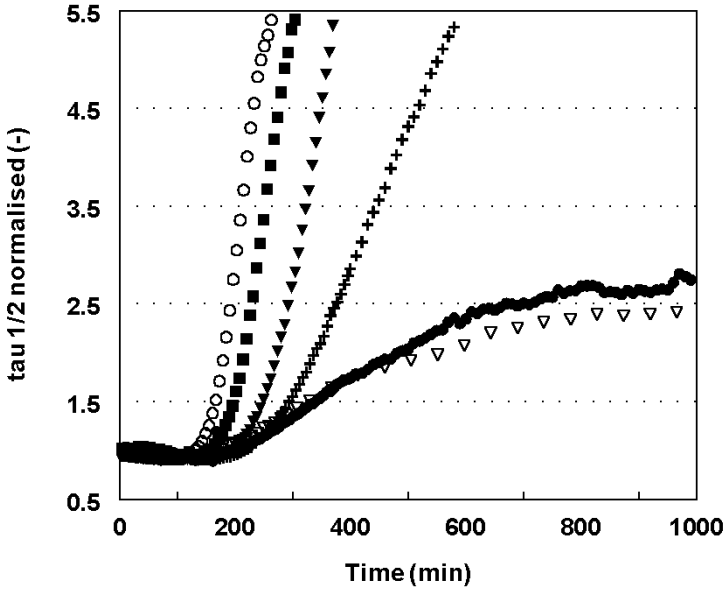

Figure 5.6: The DWS traces ($\tau_{½}$ versus renneting time) of WPF-milk with added whey proteins unheated (○) and heated for 10 min at 70 (■), 75 (▼), 80 (✚), 85 (●) and 90°C (▽) subjected to rennet treatment at 30°C.

proteins a clear effect of heat treatment was observed on clotting behaviour, while no effect at all was found in WPF-milk. Therefore, we can conclude that the impaired clotting of casein micelles in heated milk is entirely attributable to coating of the casein micelles with denatured whey proteins. This confirms one of the hypotheses often proposed in the literature (4, 13, 17, 18, 23).

Conclusions

Summarising, we can conclude that the impaired clotting of casein micelles in heated milk can be entirely attributed to whey protein denaturation. However, this denaturation does not affect the start of flocculation, but inhibits the process of clotting. No effect of calcium precipitation was found on either the enzymatic activity of rennet or the clotting of the casein micelles. The slight inhibition of enzymatic activity above 80°C in skim milk does not appear to contribute much further to the already very poor clotting properties of heated milk.

References

1. Allogio, V., F. Caponio, A. Pasqualone and T. Gommes. 2000. Effect of heat treatment on the rennet clotting time of goat and cow milk. Food Chem. 70: 51.
2. Chaplin, B. and M.L. Green. 1980. Determination of the proportion of κ-casein hydrolysed by rennet on coagulation of milk. J. of Dairy Res. 47: 351.
3. Dalgleish, D.G. 1990. The enzymatic coagulation of milk. Page 579 in Advanced Dairy Chemistry-1:proteins. P.F. Fox, ed. London Elsevier Science Publishers Ltd.
4. Dalgleish, D.G. 1990. The effect of denaturation of β-lactoglobulin on renneting - a quantitative study. Milchwissenschaft. 45: 8: 491.
5. Hindle, E.J. and J.V. Wheelock. 1970. The primary phase of rennin action in heat sterilised milk. J. Dairy Res. 37: 389.
6. van Hooydonk, A.C.M, H.G. Hagedoorn and I.J. Boerrigter. 1986. The effect of various cations on the renneting of milk. Neth. Milk Dairy J. 40: 369.
7. van Hooydonk, A.C.M., P.G. de Koster and I.J. Boerrigter. 1987. The renneting properties of heated milk. Neth. Milk Dairy J. 41: 3.
8. Jang, H.D. and H.E. Swaisgood. 1990. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. J. Dairy Sci., 73: 900.
9. de Kruif, C.G. and E.B. Zhulina. 1996. κ-casein as a polyelectrolyte brush on the surface of casein micelles. Colloids and Surfaces A. 117: 151.
10. Marshall, R.J. 1986. Increasing cheese yields by high heat treatment of milk. J. Dairy Res. 53: 313.
11. Mellema, M., F.A.M. Leermakers and C.G. de Kruif. 1999. Molecular mechanism of the renneting process of casein micelles in skim milk, examined by viscosity and light-scattering experiments and simulated by model SCF calculations. Langmuir. 15: 19: 6304.
12. Minkiewicz, P., C.J. Slangen, F.M. Lagerwerf, J. Haverkamp, H.S. Rollema and S. Visser. Reversed-phase high-performance liquid chromatographic separation of bovine κ-casein macropeptide and characterization of isolated fractions. J. Chromatography A. 743: 123.
13. Raynal, K. and F. Remeuf. 1998. The effect of heating on physicochemical and renneting properties of milk: a comparison between caprine, ovine and bovine milk. Int. Dairy J. 8: 695.
14. Reddy, I.M. and J.E. Kinsella. 1990. Interaction of β-lactoglobulin with κ-casein in micelles as assessed by chymosin hydrolysis: effect of temperature, heating time, b-lactoglobulin concentration and pH. J. Agric. Food Chem. 38: 50.
15. Schreiber, R. 2001. Heat-induced modifications in casein dispersions affecting their rennetability. Int. Dairy J. 11: 553.
16. Singh, H., S.I. Shalabi., P.F. Fox, A. Flynn and A. Barry. 1988. Rennet coagulation of heated milk: influence of pH adjustment before or after heating. J. Dairy Res. 55: 205
17. Singh, H. and A. Wauguna. 2001. Influence of heat treatment of milk on cheesemaking properties. Int. Dairy J. 11: 543.

18. Steffl, A., R. Schreiber, M. Hafenmaier and H. Kessler. 1999. Effect of denatured whey proteins on the rennet-induced aggregation of casein micelles. Int. Dairy J. 9: 401.
19. Vasbinder, A.J., P.J.J.M. van Mil, A. Bot, C.G. de Kruif. 2001. Acid-induced gelation of heat-treated milk studied by diffusing wave spectroscopy. Colloids and Surfaces B. 21: 245. Chapter 6 of this thesis.
20. Vreeman, H.J., S. Visser, C.J. Slangen and J.A.M. van Riel. 1986. Characterization of bovine κ-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. Biochem. J.240: 87.
21. Walstra, P. and T. van Vliet. 1986. The physical chemistry of curd making. Neth. Milk Dairy J. 40: 241.
22. Walstra, P. and R. Jenness. 1984. Dairy Chemistry and Physics, John Wiley and Sons, New York.
23. Wauguna, A., H. Singh and R.J. Bennett. 1996. Influence of denaturation and aggregation of β-lactoglobulin on rennet coagulation properties of skim milk and ultrafiltered milk. Food Res. Int. 29: 8: 715.
24. Wilson, G.A. and J.V. Wheelock. 1972. Factors affecting the action of rennin in heated milk. J. Dairy Res. 39: 413.
25. Wheelock, J.V. and A. Kirk. 1974. The role of β-lactoglobulin in the primary phase of rennin action on heated casein micelles and heated milk. J. Dairy Res. 41: 367.

Chapter 6: Acid-induced gelation of heat-treated milk studied by Diffusing Wave Spectroscopy

Abstract

Raw skim milk is a stable colloidal system containing casein micelles and whey proteins. By decreasing the pH the casein micelles become unstable and a gel is formed. During heat treatment at temperatures higher than 70°C the major whey proteins, e.g. α-lactalbumin and β-lactoglobulin denature and start to interact with each other and with casein micelles. This changes the colloidal properties of the casein micelles.

In this article the pH-induced gel formation of heat treated milk and the role of whey proteins was studied. Heat treatment in the range 70 to 90°C induced a shift in gelation pH of skim milk to more alkaline pH values. This shift was directly related to whey protein denaturation. By using WPF milk it was shown that β-lactoglobulin is principally responsible for the shift in gelation pH. α-lactalbumin caused neither alone nor in combination with β-lg an effect on the gelation pH. Heat treatment of milk for 10 min at 90°C resulted in complete denaturation of the β-lg present in skim milk but it is estimated that the casein micelles are coated only up to 40% by whey proteins when compared with pure whey protein aggregates.

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Introduction

The casein micelles in milk are association colloids which consist of different types of casein proteins, i.e. α_{s1}, α_{s2}, β and κ. The casein micelles also contain inorganic matter, mainly calcium phosphate, about 8 g per 100 g of casein. The micelles are sterically stabilized by the κ-casein (17, 7), which is present at the periphery of the micelle. The C-terminal part of the κ-casein molecule is very hydrophilic and it also has a considerably negative charge. Presumably, this part of the molecule sticks out partly in the surrounding medium as a "flexible" hair. De Kruif and Zhulina (4) consider the presence of the κ-casein on the surface of the micelles as a poly electrolyte brush. Any property that will effect the stability of the micelle, e.g. ethanol, rennet, acid, will markedly destabilise the poly electrolyte brush and ultimately cause coagulation (6, 8, 11, 17).

On heat treatment at temperatures higher than 70°C the major whey proteins i.e. β-lactoglobulin (β-lg) and α-lactalbumin (α-lac) denature (3), but β-lg denatures at a much higher reaction rate than α-lac. It is well known that β-lg interacts with casein micelles involving κ-casein when milk is heated at temperatures above 70°C (9, 15). The whey proteins start to interact with each other and with casein micelles, which changes the colloidal properties of the casein micelles (see Figure 6.1).

In the dairy industry the acidification of milk takes usually place by lactic acid bacteria. However, for model systems it is much

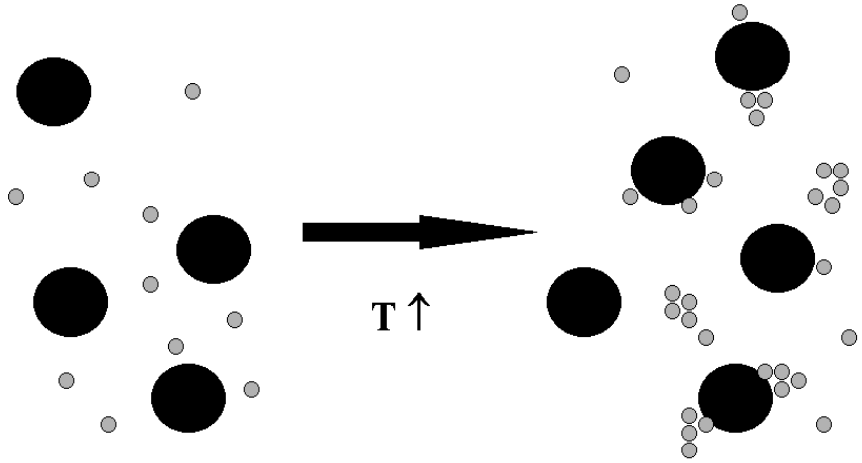

Figure 6.1: Schematic representation of the heat treatment of milk showing casein micelles (large spheres) and whey proteins (small dots)

easier to mimic this process by the addition of glucono δ lacton (GDL); an ester that slowly hydrolyses to produce a weak acid (gluconic acid).

During the acidification of milk the mobility of the particles changes, particularly around the gelation point where the casein micelles start to aggregate. Diffusing Wave Spectroscopy (DWS) is used for continuous monitoring of the coagulation process of the casein micelles during acidification. DWS analyses fluctuations in light back scattered from the sample using correlation techniques to determine a characteristic relaxation time for the mobility of colloidal particles in the system (6, 14). It is therefore a very suitable technique to investigate the influence of process parameters on the gel point. After gelation the particle network still exhibits a thermally driven motion, counteracted by the visco-elasticity of the gel network. As a result the scattered light intensity still fluctuates but now contains information on the visco-elasticity of the gel (4).

In this article we studied the acid-induced gel formation of heat-treated milk by using DWS. Combination of the DWS technique and the use of whey protein free-milk allowed us to determine which whey protein is responsible for the interaction with casein micelles during heat treatment. This is a rather new approach to look at protein interaction mechanisms in milk as until now mostly analytical techniques have been used such as chromatographic and electrophoretic techniques either alone or in combination with ultracentrifugation (1, 2, 12, 16). Furthermore the pH at which gel formation occurs was related to the degree of coating of the micelles. This revealed that casein micelles seem to be coated only partially after a heat treatment even if all the whey proteins are denatured.

Materials and methods

Skimmed milk and Whey Protein Free milk

Low-heat skim milk powder (Nilac; NIZO food research Ede) was prepared by dissolving 10.45 gram of milk powder in 100 g distilled water while gently stirring. Whey protein free (WPF) milk (microfiltration / UF; NIZO food research) was prepared by dissolving 8.95 g of WPF milk powder in 91.05 gram distilled water (8.95%, w/w). The milk was stirred at 45°C for 1 hour. To prevent bacterial growth 0.02% sodium azide was added and kept overnight at 4°C before use. The initial pH of the milk was 6.67 (± 0.01). The

composition of these milks is discussed in the materials and methods section of chapter 2.

α-lactalbumin and β-lactoglobulin solution

These powders were obtained by ion exchange chromatography on a Staeck system (Pharmacia). An 8.8% (w/w) β-lg and a 10% (w/w) α-lac solution were prepared by dissolving freeze dried powders in distilled water at 25°C. The solutions were stirred until dissolved and filtered (0.1 μm).

WPF-milk with addition of α-lac and β-lg

The WPF-milk was prepared at a slightly higher concentration (8.95% (w/w)) than the finally desired (8.42% (w/w)), which was obtained by mixing 14.1 g WPF milk with 0.9 g of distilled water or the amounts of α-lac and β-lg solutions as required.

Sample preparation

The required amount of skim milk or WPF-milk (stored at 4°C), with or without added whey proteins, was stirred for 2 hours at 20°C. Reconstituted skim milk and reconstituted WPF milk enriched with α-lac and β-lg were heat treated for 10 min in aliquots of 5 ml. After heat treatment at temperatures in the range 70-90°C the samples were kept at 32°C for 75 min and then acidified with 1.1% GDL (Sigma Chemical Co.) to pH ~ 4.6 at 32°C. After addition of GDL the milk was stirred gently for 2 min. Gelation was followed by DWS and pH measurements as function of time.

Diffusing Wave Spectroscopy

Light from a 5 mW He-Ne laser set at 632.8 nm was passed through a multi mode fiber into the milk. The back scattered light was monitored by a single mode fiber located between 1.8 and 3.5 mm from the input fiber. The scattered light is detected with a Photo Multiplier Tube (ALV SO-SIPD), transforming the light signal into an electronic signal, which is fed to a PC interfaced autocorrelator board (ALV5000/E). The time where the auto correlation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{½}$ (see Figure 6.2). The gelation point is defined in the plot of $\tau_{½}$ against pH at the

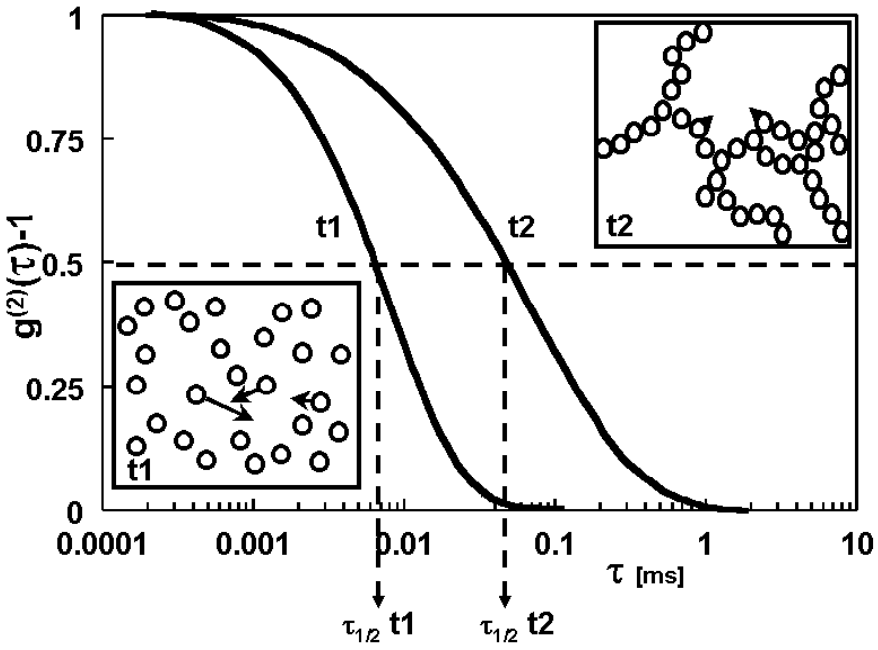

Figure 6.2: Schematic representation of the determination of $\tau_{½}$ in the auto correlation curve and its dependence on the "mobility" of the system; t1 liquid state, t2 gel state

inflexion point. All data were normalised relative to its control blank, which is the sample prior to GDL addition.

Preparation of β-lg aggregates

A solution containing 3.0% β-lg in 60mM NaCl at pH 7.0 was heated for 12 hours at 68.5°C. After cooling the volume was reduced to a protein concentration of 3.7% by using centripep YM-3 concentrators (Amicon Bioseparators; nominal molecular weight cut off of 3000 Da). Concentrated simulated milk ultrafiltrate (SMUF(10); 5.5 times concentrated) was added so that a protein concentration of 3.3% was obtained (to mimic the salt and protein concentration in skim milk 2.76% casein + 0.53% whey = 3.29% total protein). After filtration (5 μm) the size of the aggregates was determined by dynamic light scattering and was 225 nm. This solution is acidified with 0.5% (w/w) GDL at 32°C.

Determination of native protein

An amount of 0.4 g of milk was mixed with 0.8 g of distilled water (40°C) and 40 μl acetic acid (HAc) (10%) in an eppendorf tube (2

ml). After mixing (vortex) and 10 min waiting, 40 µl of sodium acetate (NaAc; 1M) and 0.72 g of distilled water are added and the solution was mixed again. After 1 hour standing the solution is centrifuged for 5 min at 3000g. The concentration of native β-lg and α-lac in the supernatant is determined by chromatographic analysis.

Results and discussion

Figure 6.3 shows the effect of heat treatment on the acid induced gelation of milk measured by DWS and plotted as $\tau_{1/2}$ as a function of pH. The $\tau_{1/2}$ is the time where the intensity autocorrelation function has decayed for 50% and thus represents a " mobility" of the system. The value of $\tau_{1/2}$ correlates in a one to one manner with G" (ω=1s^{-1}), where G" is the small deformation loss modulus of the system. The milk at its natural pH was heated, in the temperature range 70-90°C for 10 min. The milk was cooled down to 32°C, the acidification temperature, and after 75 min acidified with 1.1% GDL. Heat treatment of the milk, prior to acidification, induced a shift of the gelation pH to more alkaline pH values. These results are in agreement with the results of Horne and Davidson (6).

Heat treatment of skim milk in the range 70-90°C will result in

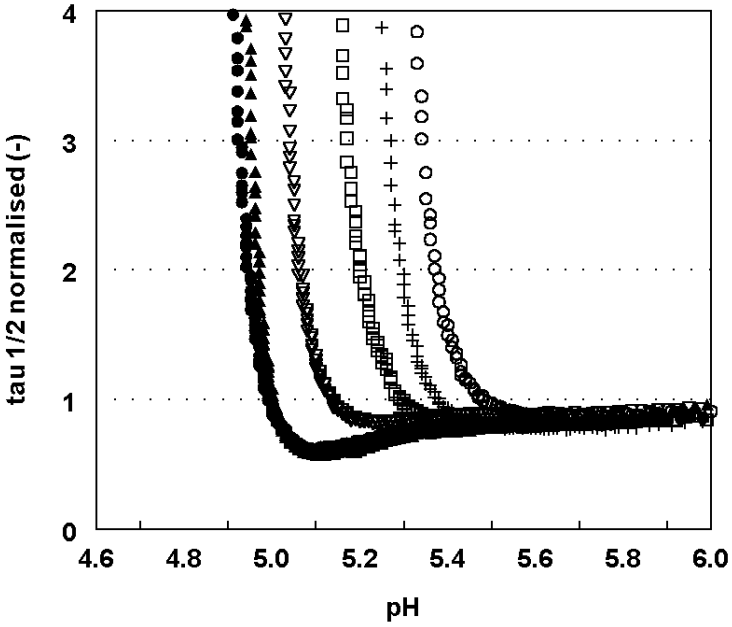

Figure 6.3: Acidification traces, normalised $\tau_{1/2}$ versus pH, of skim milk heat treated for 10 min at 70(●), 75(▽), 80(□), 85(+), 90(○)°C and unheated (▲) with 1.1 % GDL at 32°C

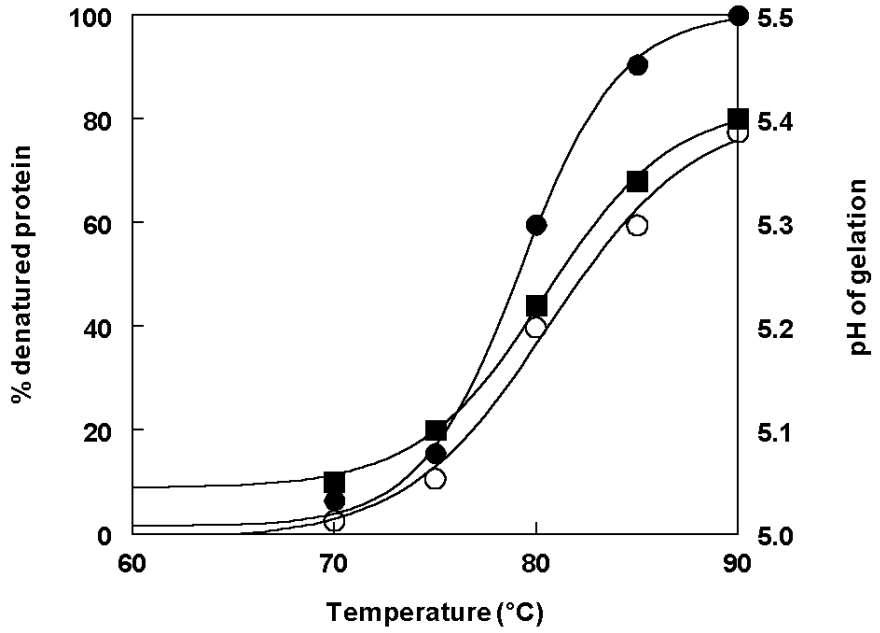

Figure 6.4: Percentage of denatured whey protein; β-lg (●) and α-lac (○) in skim milk. Gelation pH (■) measured by DWS during acidification with 1.1% GDL at 32°C as function of the heat treatment temperature

whey protein thermal denaturation (3). The results as shown in Figure 6.3 are related to the thermal denaturation of α-lac and β-lg in the milk prior to acidification. Figure 6.4 shows an intrinsic relationship between the extent in whey protein thermal denaturation and the pH at which gelation occurs.

In order to ascertain the protein responsible for the shift in gelation pH, the effect of adding α-lac and/or β-lg to WPF-milk was investigated (Figure 6.5). The $\tau_{½}$-pH traces of WPF-milk with additions of α-lac and/or β-lg but without further heat treatment of the milk were all identical to untreated WPF-milk (results not shown). As shown in figure 6.5 heat treatment had no effect on the gelation pH of WPF. This supports the earlier assertion that the whey proteins are responsible for the shift of the gelation pH to more alkaline pH values. Heat treatment had little effect on the gelation pH of WPF with or without α-lac supplementation. Addition of β-lg caused gelation to occur at a more alkaline pH. This is consistent with literature as it is known that β-lg interacts with κ-casein by intermolecular disulfide bonds. α-lac has no free thiol group and is therefore not able to interact with κ-casein (13, 17). Supplementation of both α-lac and β-lg induced no additional shift to β-lg alone, although a large amount of α-lac is denatured in the presence of β-lg, but apparently this extra denaturation does not influence the gelation

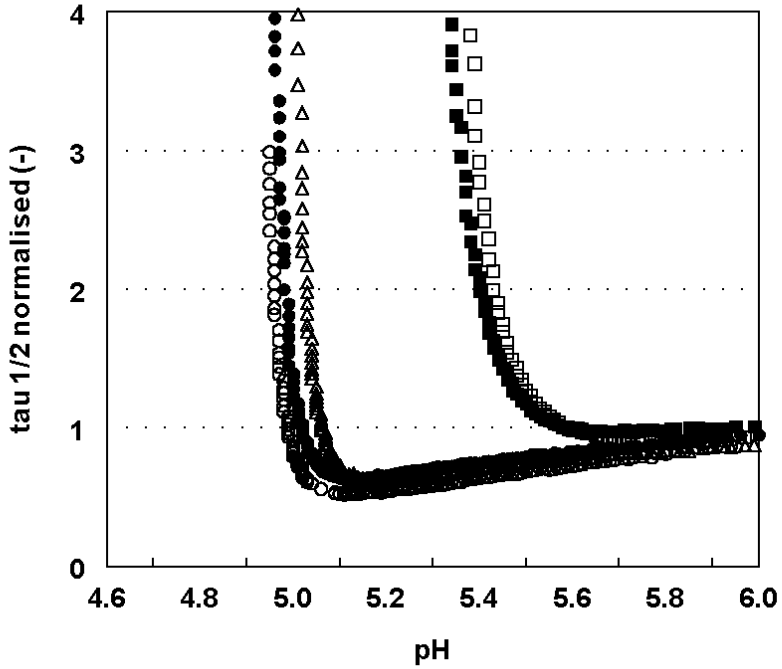

Figure 6.5: Acidification traces, normalised $\tau_{½}$ versus pH, of heat treated (10 min, 90°C) WPF-milk (●), WPF-milk + 0.12% α-lac (Δ), WPF-milk + 0.32% β-lg (■), WPF-milk + 0.12% α-lac + 0.32% β-lg (□) and WPF-milk unheated (○)

pH. The overall conclusion is that β-lg is responsible for the shift in gelation pH which was already noticed in milk and that α-lac neither alone nor together with β-lg has any (additional) influence on the gelation pH.

The addition of β-lg to WPF to an equal concentration as in milk induced a shift of 0.5 pH unit to more alkaline pH. The role of β-lg was further explored by progressive addition to WPF-milk. The gelation pH values are obtained from the $\tau_{½}$ acidification curves and plotted versus the total amount of denatured β-lg. All the milks were heated for 10 min at 90°C which caused 100% denaturation. Figure 6.6 confirms the role of β-lg by the observation that with the progressive addition of β-lg up to 4 g/l there is a concomitant increase in gelation pH; thereafter the gelation pH levels off.

Whey proteins are thought to interact with κ-casein during heat treatment (9, 15) and thus form a coating on the micelle surface. The properties of the micelles are changed by the coating with whey proteins as the pI of whey proteins is markedly higher than that of the caseins (5.2; 4.6 respectively). This raises the question, in a colloidal context, to what extent do whey protein coated casein micelles start to behave like whey protein particles. To obtain this

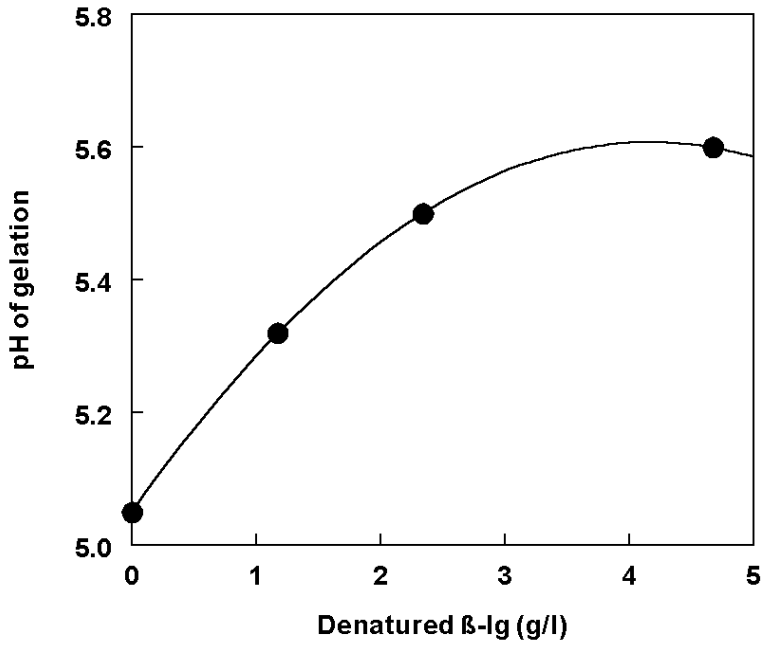

Figure 6.6: Effect of β-lg denaturation on the acid-induced gelation point of WPF milk containing 0-5 g/l β-lg, heated (10 min, 90°C) and acidified with 1.1% GDL at 32°C.

information the gelation pH of non-heat treated casein micelles (1.1% GDL, 32°C) and β-lg aggregates ~225nm in SMUF (0.5% GDL, 32°C), assumed as 0 and 100% coating, respectively was determined. These β-lg aggregates were prepared in order to mimic a completely coated micelle with roughly the same size as a micelle (225 nm). The aggregates were acidified in SMUF to simulate a milk milieu. The gelation pH of the β-lactoglobulin aggregates were taken as $pH_{(100\% coating)}$; pH = 6.00. For non coated casein micelles (WPF) the gelation pH is 5.05. It was assumed (see figure 6.7) that gelation pH is linearly related to the coverage of the micelles. It was then found that the heat treated micelles (10 min at 90°C; gelation pH is 5.40) are covered by 40% by whey proteins. Surprisingly this seems to be a very reasonable estimate, as calculating shows.

The amount of β-lg required to form a monolayer on the surface of the micelles present in milk was calculated. The following parameters were taken: ϕ = 0.11; ϕmax = 0.63; rmicel = 100 nm (ϕ=volume fraction [-]) and a mono layer is formed at 2 mg/m2. By using ϕ = (number * volume) / total volume it was calculated that in case of monodispersity 6 g/l could attach to casein micelle to form a mono layer. In milk there is 3.2 g/l β-lg, which means that not even a mono layer can be formed. The same qualitative result is obtained by the fact that β-lg and κ-casein are present in milk at an equal

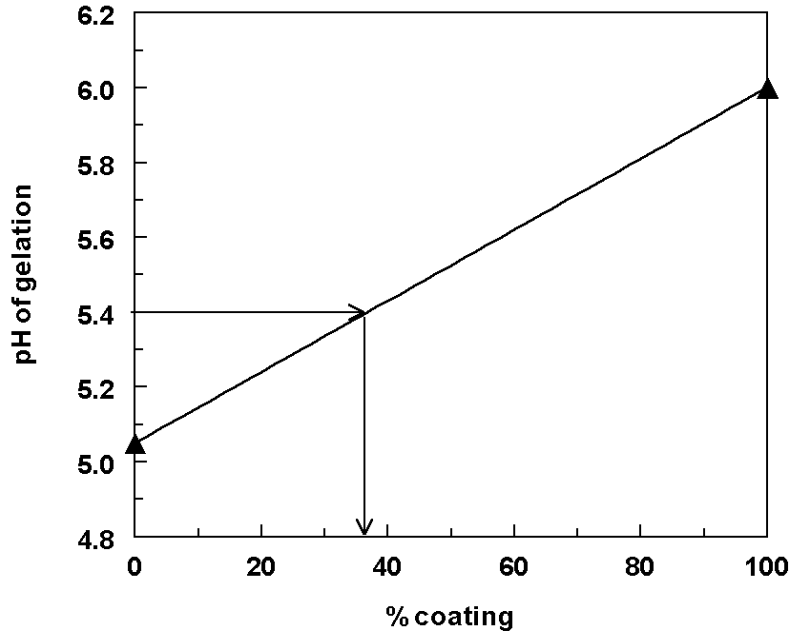

Figure 6.7: An estimate to relate the measured gelation pH to the degree of coating of micelles. Two gelation points are measured: non heat treated casein micelles (1.1% GDL, 32°C) and β-lg aggregates ~225nm in SMUF (0.5% GDL, 32°C), assumed as 0 and 100% coating, respectively. The arrows indicate the measured gelation pH of skim milk heat treated for 10 min at 90°C and the estimated degree of coating.

molarity of 180 mmol/m3 (17). A small part of the κ-casein is not present at the pheriphery but in the enterior of the micelle (17), which would mean there is hardly enough to get a mono layer. We also have to take into account that during heat treatment β-lg will also form small aggregates by interacting with α-lac and other β-lg molecules. The aggregates will mainly interact with the micelles, but we have indications that a part stays in solution. The amount of available β-lg to coat the micelle by a mono layer is reduced by the formation of these aggregates. Considering the above it is concluded that whey protein coated micelles in milk only behave for 40% like "pure" whey protein aggregates.

Conclusions

Heat treatment in the range 70 to 90°C induced a shift in gelation pH of skim milk to more alkaline pH values. This shift was directly related to whey protein denaturation. By using WPF milk it was shown that β-lactoglobulin is principally responsible for the shift in gelation pH. α-lactalbumin caused neither alone nor in combination with β-lg an effect on the gelation pH. Heat treatment of

milk for 10 min at 90°C resulted in complete denaturation of the β-lg present in skim milk but it is estimated that the casein micelles are coated only up to 40% by whey proteins when compared with pure whey protein aggregates.

References

1. Corredig, M. and D.G. Dalgleish. 1996. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Res. Int. 29: 1: 49.
2. Dalgleish, D.G., L. van Mourik, M. Corredig. 1997. Heat-induced interactions of whey proteins and casein micelles with different concentrations of α-lactalbumin and β-lactoglobulin. J. Agric. Food Chem. 45: 4806.
3. Dannenberg, H. and H.G. Kessler. 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 1. Syneresis. Milchwissenschaft. 43: 632.
4. Gisler, T. and D.A. Weitz. 1999. Scaling of the microrheology of semidilute F-actin solutions. Physical Review Letters, 82: 1606.
5. Horne, D.S. and C.M. Davidson. 1993. Influence of heat treatment on gel formation in acidified milks. Protein and fat globule modification, in Proceedings of IDF Seminar. 267.
6. Horne, D.S. and C.M. Davidson. 1986. The effect of environmental conditions on the steric stabilization of casein micelles. Colloid Polymer Sci. 264: 727.
7. Holt, C. 1992. Structure and stability of bovine casein micelles. Advances in Prot. Chem. 43: 63.
8. Holt, C. and D.S. Horne. 1996. The hairy casein micelle: Evolution of the concept and its implications for dairy technology. Neth. Milk Dairy J. 50: 85.
9. Jang, H.D. and H.E. Swaisgood. 1990. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. J. Dairy Sci. 73: 900.
10. Jenness, R., J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. Neth. Milk Dairy J. 16: 153.
11. de Kruif, C.G. and E.B. Zhulina. 1996. κ-casein as a polyelectrolyte brush on the surface of casein micelles. Colloids and Surfaces A. 117: 151.
12. Law, A.J.R., D.S. Horne, J.M. Banks and J. Leaver. 1994. Heat-induced changes in the whey proteins and caseins. Milchwissenschaft. 49: 3: 125.
13. Lucey, J.A., M. Tamehana, H. Singh and P.A. Munro. 1998. Effect of interactions between denatured whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. J. Dairy Res. 65: 555.
14. Maret, G. 1997. Diffusing Wave Spectroscopy. Current Opinion Coll. Interface Sci. 2: 251.
15. Singh, H.S. 1993. Heat-induced interactions of proteins in milk. Protein and fat globule modifications-IDF seminar, special issue 9303. 191.
16. Smits, P. and J.H. van Brouwershaven. 1980. Heat induced association of β-lactoglobulin and casein micelles. J. Dairy Res. 47: 313.

17. Walstra, P. and R. Jenness. 1984. Dairy Chemistry and Physics, John Wiley and sons, Inc, USA.

Chapter 7: Texture of acid milk gels: formation of disulfide cross-links during acidification

Abstract

Denaturation of whey proteins during pasteurisation of milk results in the formation of whey protein aggregates and whey protein coated casein micelles. After cooling a substantial number of thiol groups remains exposed. Formation of larger disulfide linked protein structures during acidification at ambient temperature was demonstrated by analytical methods. The time dependent formation of these structures attributed significantly to the mechanical properties of acid milk gels, resulting in gels with an increased storage modulus and hardness. Addition of the thiol blocking agent N-ethylmaleimide prevented the formation of disulfide-linked structures. The mechanical properties are shown to be the result of the contribution of denatured whey proteins to the protein network as such and the additional formation of disulfide bonds. Surprisingly, the formation of these disulfide bonds take place at ambient temperature and under acidic conditions. Therefore, the disulfide cross-linking is highly relevant for textural properties of acid-milk products, like yogurt.

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Introduction

Historically, acidification of heated milk was applied as a preservation technique. Nowadays, acidified products like yogurt are highly appreciated for their texture, taste and health properties. During yogurt preparation, the milk is subjected to a heat treatment before acidification. Therefore, production of yogurt can be seen as a two step process, which is schematically depicted in Figure 7.1. Step 1 (T ↑) represents the heat treatment of skim milk during which denaturation of whey proteins occurs at temperatures in the range of 70 to 90°C. Step 2 (pH ↓) illustrates the acidification step performed after the milk has been cooled, which causes clotting of the casein micelles and whey protein aggregates into a protein network.

Heat treatment of milk (step 1, Figure 7.1)

On heat treatment at temperatures higher than 70°C the major whey proteins, i.e. β-lactoglobulin (β-lg) and α-lactalbumin (α-lac), denature (5). The temperature induces conformational change of β-lg results in the exposure of both hydrophobic parts of the polypeptide and reactive thiol groups. These reactive thiol groups can form disulfide links with other reactive thiol groups or disulfide

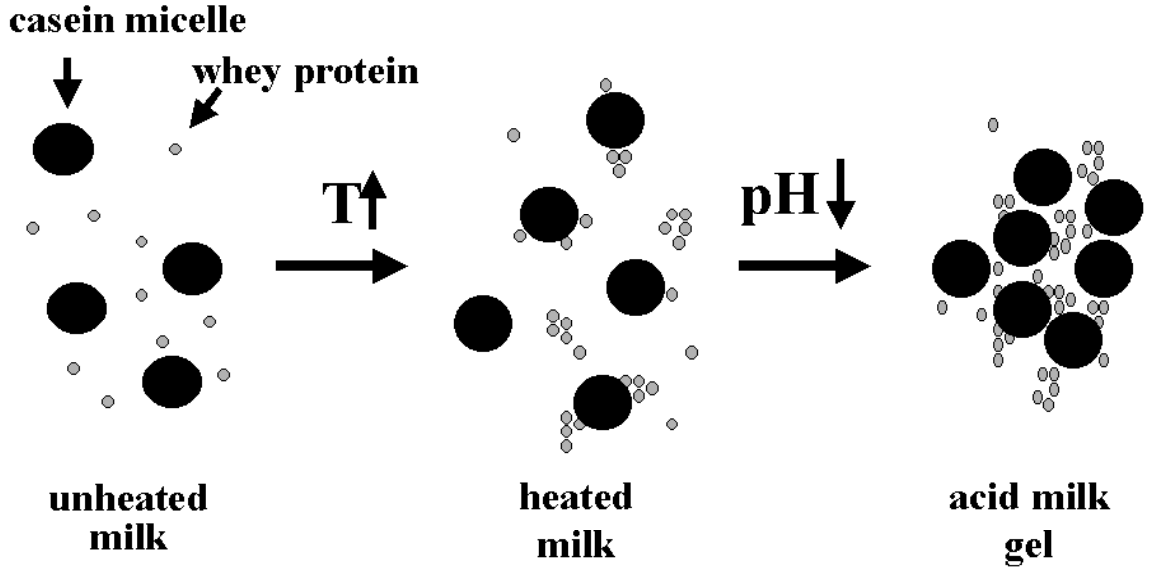

Figure 7.1: Schematic representation of the heating of skim milk and the subsequent acidification resulting in the formation of a protein network. The small circles represent the whey proteins, the large circles the casein micelles.

bridges as present in α-lac, β-lg, bovine serum albumin, κ- and α_{s2}-casein through thiol group/disulfide bond interchange reactions. During the heating of milk β-lg covalently interacts mainly with κ-casein present at the exterior of the casein micelles (4, 16, 30). Additionally, soluble disulfide linked whey protein aggregates are formed (2, 24). So, heated milk is a complex mixture of native and denatured whey proteins and casein micelles in which the denatured whey proteins occur either as whey protein aggregates or as a coating present on casein micelles. A significant amount of thiol groups will remain active after cooling the milk to ambient temperature (11, 12, 14, 17, 18). It was reported that a maximum number of free thiols can be determined after heat treatment at 90°C (10 min). A longer heat treatment resulted in a steady decrease probably due to oxidation (12, 17, 18).

Acidification of heated milk (Step 2, Figure 7.1)

Effect of the presence of whey proteins

Acidification of milk towards the iso-electric point of the caseins, i.e. pH 4.6, lowers the stability of the κ-casein brush on the surface of the casein micelles (31). The casein micelles loose their steric stabilization and Van der Waals attraction causes flocculation. Acidification of heated milk at temperatures ranging from 20 to 40°C changes the gelation properties markedly compared to those of unheated milk. Heat treatment had caused a shift in gelation pH towards higher pH values (13, 15, 21, 34). The final gel formed has an increased gel hardness, higher storage modulus (G') (20, 21, 25, 36) and shows less susceptibility to syneresis (6, 7). These effects are related to whey protein denaturation and the whey protein coating of the casein micelles. Due to the coating with whey proteins the pH at which the casein micelles tend to neutrality (pH 4.6) is shifted to pH 5.2, which is the pI of β-lg (34). Electron microscopy revealed that heat treatment of milk changed casein micelles into micelles with appendages composed of whey proteins on the surface. These whey protein coated micelles are thought to cause the increased gel hardness and decreased syneresis as they prevent coalescence of the micelles and increase the number of contact points between the micelles (8, 13, 23).

Effect of thiol group / disulfide bond interchange reactions

It seems likely that the exposed thiol groups formed during heat treatment, and still detectable after cooling, have an additional effect on gel strength. Lucey et al. (20) demonstrated that addition of a thiol blocking agent, N-ethylmaleimide (NEM), after heat treatment, but prior to acidification at 30°C, caused a decrease of 15% in the final storage modulus (G'). It was not further investigated whether this was due to inhibition of thiol group/disulfide bond interchange reactions or non-specific interference of NEM with gel formation (20). As far as we know this is the only experiment performed under conditions close to ambient temperature. Hashizume and Sato (12) and Goddard (10) performed more extensive studies on the relation between thiol groups and gel strength, but the acid coagulation took place at 60°C (Goddard, 1996) or at temperatures between 60 and 80°C (12). Goddard (10) demonstrated that addition of more than 0.05mM NEM to heated milk prior to acidification decreases the storage modulus by 35% to a plateau value, while addition of mercaptoethanol, which provides a free thiol group that can initiate β-lactoglobulin aggregation, increased the storage modulus of yogurt like gels. Hashizume and Sato (12) observed that higher acidification temperatures (60 to 80°C) have larger effects on gel strength. The solubility of these gels in urea buffer with and without mercaptoethanol demonstrated the formation of disulfide linked structures in the gel state.

Hashizume and Sato (12) further demonstrated that thiol group/disulfide bond interchange reactions took place at a faster rate and to a larger extent during acidification at higher temperatures (60°C compared to 80°C). At these temperatures the processes of whey protein denaturation and acid-induced gelation are intertwined. Additionally, these temperatures are much too high for lactic acid bacteria to fulfill their essential role in the production of acidified milk products, i.e. production of lactic acid and extracellular polysaccharides. In daily practice, production of yogurt takes place at far lower temperatures (20-40°C); here it is a two step process where denaturation and acid-induced gelation are clearly separated. Therefore, it is questionable whether the disulfide exchange reactions observed by both Hashizume and Sato (12) and Goddard (10) will still take place at an acidification temperature of 20°C and at a time scale relevant for yogurt production, i.e. 24 to 48 hours.

In this article we demonstrate the presence of free thiol groups in heated milk and the time dependent development of disulfide bridges during gelation (20°C). The formation of disulfide linked structures during gelation was shown as well as their effect on the mechanical properties of acid milk gels. This work contributes to a better understanding of thiol group/disulfide bond interchange reactions during gel formation under temperature conditions relevant for the production of yogurt and demonstrates their importance for texture formation.

Material and Methods

Reagents and chemicals

Glucono-δ-lactone (GDL), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulphate (SDS), dithiothreithol (DTT), and N-ethylmaleimide (NEM) were obtained from Sigma Chemicals (St. Louis, MO. USA). Electrophoresis-grade agarose was obtained from Life Technologies (Paisley, Scotland). Phastgel blue R tablets were from Pharmacia Biotech (Uppsala, Sweden). Fresh skim milk was obtained from the NIZO pilot plant (Ede, the Netherlands). Transglutaminase (Ca^{2+}-independent) was supplied by Ajinomoto Co. Inc. (Japan).

Preparation of reconstituted skimmed milk

Reconstituted skim milk was prepared by dissolving 10.45 g milk powder (Nilac; NIZO food research, Ede) in 100 g distilled water while gently stirring. The milk was stirred at 45°C for 1 hour. To prevent bacterial growth 0.02% sodium azide was added and the milk was kept overnight at 4°C before use. The initial pH of the milk was 6.67 (± 0.01). The composition is discussed in the materials and methods section of chapter 2.

Preparation of whey protein free reconstituted skim milk

Whey protein free reconstituted skim milk was prepared by dissolving 8.78 g whey protein free milk powder (prepared by ultra- and micro-filtration) in 91.22 g distilled water. Further treatment was identical to preparation of skimmed milk as described above. The

composition is discussed in the materials and methods section of chapter 2.

Intra-micellar cross-linking of the micelles by transglutaminase

The required amount of reconstituted skim milk (stored at 4°C) was stirred for 2 hours at 20°C and then incubated for 1hours at 40°C. A 2% transglutaminase solution (activity 20 units g^{-1}) was used to reach a final activity in the milk of 50U g^{-1} protein (protein content of milk is 3.5%). This solution was mixed and incubated for 1 hour at 40°C. The solution was transferred into glass tubes (5 mL per tube) and heat treated for 25 min at 90°C. After cooling under tap water to 20°C the milk was either used directly or stored overnight at 4°C.

Sample preparation: heat treatment

The required amount of fresh and reconstituted skim milk and whey protein free reconstituted skim milk (stored at 4°C) was stirred for 2 hours at 20°C, followed by a heat treatment of 10 min at 90°C in aliquots of 5 mL. After heating the samples were cooled under tap water.

Blocking and determination of reactive thiol groups

The number of accessible thiol groups in milk was determined essentially according to Hashizume and Sato (12), using DTNB, also known as Ellman's reagent (9). Shortly, to 5 mL milk, 4 mL of 50 mM imidazol-buffer pH 7 was added and 1 mL of a 1 mg ml^{-1} solution of DTNB in the same buffer. After 5 min, 4 g of ammonium sulfate was added to coagulate the milk proteins that were subsequently separated by centrifugation. From the extinction at 412 nm, the number of thiol groups was calculated using a molar extinction coefficient for 2-nitro-5-mercaptobenzoic acid of 13,600 M^{-1} cm^{-1}. The assay was performed in the absence of detergents like urea or SDS, since under these conditions only the thiol groups of interest, those at the surface of the (coated) micelles, were determined. To block the thiol groups in heated milk, NEM was added in a final concentration of 5 mM. The effectiveness of this treatment was confirmed as described above.

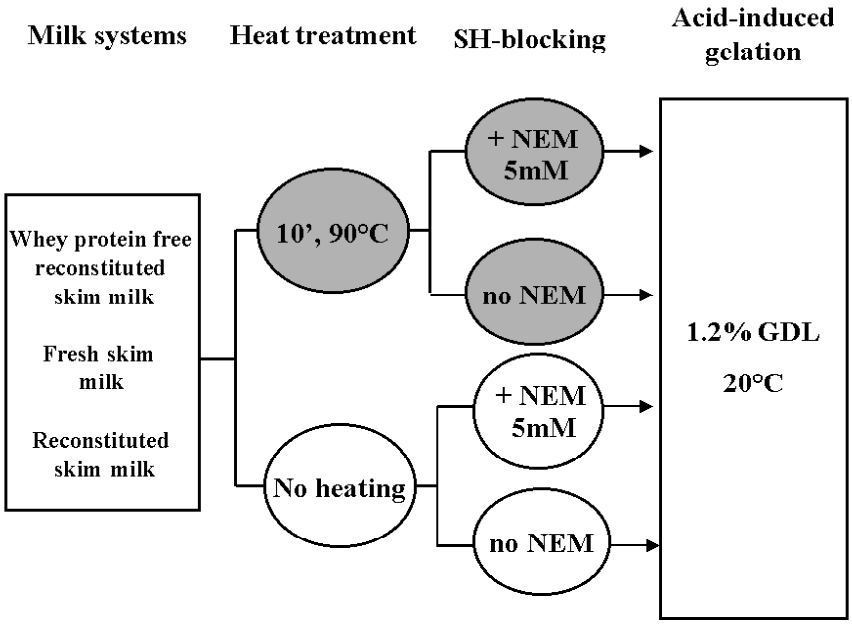

Figure 7.2: Flow scheme of the treatment of the different types of milk.

Sample preparation: acidification by GDL in absence or presence of NEM

The different milks were kept at 20°C for 75 min. NEM was added in a final concentration of 5mM prior to acidification with 1.2% GDL. After addition of GDL the milk was stirred gently for 2 min and incubated at 20°C. A pH of around 4.6 is reached after approximately 24 h. This acidification procedure is schematically depicted in Figure 7.2.

Solubilization of acid-induced gels

Solubilization of the acid-induced gels was done according to Alting et al. (1). After 24 hours of incubation with GDL the gel formed was mixed with 3 parts (w/w) of 20 mM Bis-Tris buffer and 5% SDS (pH 7.0). The samples were held overnight at ambient temperature, while being constantly stirred. In some experiments a treatment with the disulfide-reducing agent DTT (0.05%) was carried out to reduce all the disulfide bonds in solubilized samples.

Agarose gel electrophoresis

SDS-agarose gels were prepared with 0.7% agarose for milk samples and 0.4% agarose for cross-linked systems. The agarose gel

electrophoresis was carried out according to Alting et al. (1). Briefly the electrophoresis buffer consisted of 100 mM Tris, 50 mM sodium acetate, 2 mM EDTA and 0.1% SDS, and was brought to pH 7.9 with concentrated acetic acid. The milk samples and resolubilised gel samples were mixed with 1 part of 20 mM Bis-Tris buffer, 5% SDS (pH 7.0). The samples were held overnight at ambient temperature. Prior to electrophoresis, 5% of a solution containing 60% glycerol, and 0.002% bromophenol blue was added. The gels were run with a constant voltage of 50V for approximately 2 hours and stained with Phastgel blue R.

Gel hardness

Gel hardness was determined with a texture analyser (type TA-XT2, Stable Micro Systems ltd., Godalming, England). The acidification took place in a beaker containing 100 g milk. After 24 hours of incubation, the acid-induced gels were penetrated with a wire mesh-device. The mesh consisted of four blades (45 x 1.5 x 2 mm) of stainless steel arranged in a double cross. A force-time curve was obtained at a crosshead speed of 0.3 mm s^{-1} for a 10 mm displacement, and gel hardness was expressed as the force (g) at the maximum peak of the force-time curve (3).

Storage modulus G'

The storage modulus G' was determined with a Carrimed Rheometer (type CSL^2 500, TA instruments N.V./S.A.-Benelux). The acidification took place in the rheometer at 20°C and G' was followed with time. Measurements were carried out every 12 min at 1 rad s^{-1} and at a constant strain of 1%, which is in the linear region.

Confocal scanning laser microscopy

Imaging was performed using a Leica confocal scanning laser microscope, type TCS-SP, configured with an inverted microscope, and an Ar-Kr laser for single-photon excitation. The protein gels were stained by applying 2 µL of an aqueous solution of 0.05% Rhodamine B. The 568 nm laser line was used for excitation, inducing a fluorescent emission of Rhodamine B, detected between 600 and 700 nm.

Dynamic light scattering experiments

Dynamic light scattering experiments were performed as outlined by Verheul et al. (35) using a Malvern Autosizer IIC Submicron Particle Size Distribution Analyzer. The system consisted of a Malvern PCS41 optics unit with a 5 mW He-Ne laser, and a Malvern K7032-ES correlator used in serial configuration. The Autosizer IIC worked at a fixed scattering angle of 90^{o} and the wavelength of the laser beam was 632.8 nm. Both the scattering intensity and the apparent diameter were evaluated. The quartz cuvette (10 mm) containing the sample was thermostatted by a Joule-Peltier thermostat (20 ^{o}C). The apparent diameter of the micelles in solution was calculated from a cumulant fit of the intensity autocorrelation function. Before analysis, samples were filtered through a low-protein-binding membrane (5 μm; Millex-SV, Millipore Corporation, Bedford, MA., USA).

Results and Discussion

Formation of reactive thiol groups during heat treatment of milk

A heat treatment of 10 min at 90^{o}C caused more than 90% denaturation of the whey proteins (34) and resulted in a significant exposure of reactive thiol groups in reconstituted and fresh skim milk, respectively 0.07 and 0.10 mM. In the whey protein free reconstituted skim milk thiol groups were not detectable after the same heat treatment. Addition of 5 mM NEM to the different types of milk resulted in a total blocking of the thiol groups, as the absorbance measured at 412 nm was identical to the unheated milk samples.

In agreement with previously reported work (12, 14, 17, 18, 26) heat treatment of milk caused the exposure of reactive thiol groups. The higher number of thiol groups determined in fresh skim milk is probably due to the additional spray drying process in the case of reconstituted skim milk. As reported previously by Pofahl and Vakaleris (26), in the absence of whey proteins (whey protein free milk) only a negligible number of free thiol groups could be detected after 10 min of heat treatment. The results demonstrate the relevance of whey proteins in the formation of reactive thiol groups during heating of milk.

Addition of 5 mM NEM after the heat treatment and prior to acidification was sufficient to block all detectable thiol groups in the different types of heated milk studied. In the literature different concentrations of NEM in milk are proposed to inactivate the thiol groups formed during heat treatment. Lucey et al. (20) reported a relatively high concentration of 20 mM. However, a much lower quantity of NEM was reported by Goddard (10), who demonstrated that a concentration of 0.075 mM NEM was sufficient to exclude the effect of disulfide formation on the gel strength. Alting et al. (1) observed that 0.5 mM NEM was sufficient to block a concentration of 0.35 mM thiol groups in a solution of whey protein aggregates. The number of detectable thiol groups in the present milk systems is only a fraction of this.

Effect of heat treatment on the casein micelle system

In order to investigate the effect of heating on the casein micelle structure and subsequently on the mechanical properties after acidification, large deformation experiments with acidified reconstituted skim milk gels in the absence of whey proteins were performed. Figure 7.3 clearly shows that the force-distance curves for wire mesh penetration for unheated and heated whey protein free reconstituted acid skim milk gels are identical. In this study no effect of heat treatment was observed on the large deformation studies performed on acid gels of whey protein free milk. Diffusing wave spectroscopy experiments had already shown that the start of gelation of whey protein free milk was not affected by a similar heat treatment (34). Therefore we can conclude that in this study the gel formation and mechanical properties of casein-micelle based gels are not affected by heat-induced changes in the casein micelle system (29).

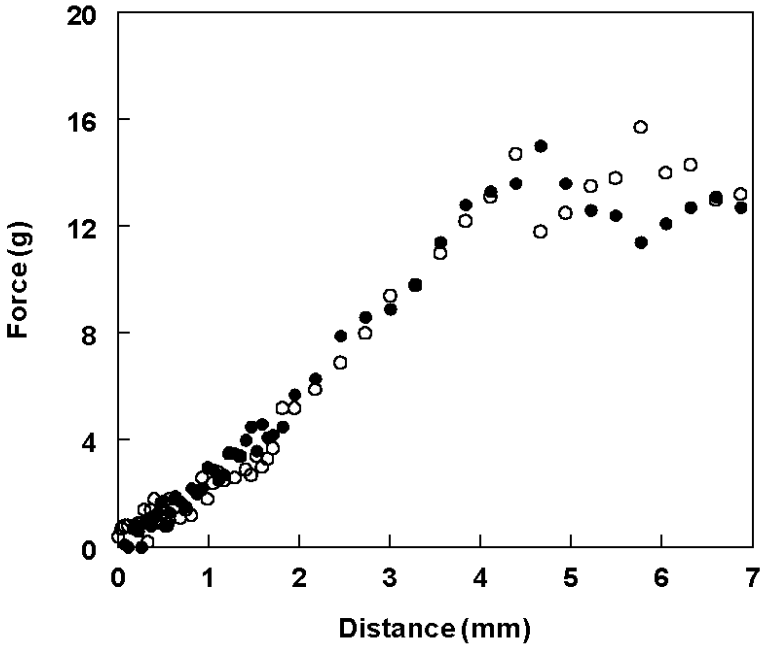

Figure 7.3: Effect of heat treatment on the large deformation properties of whey protein free reconstituted skim milk in the absence (open circles) and presence of NEM (closed circles) after 24 hours of acidification.

Effects of NEM on mechanical properties of acid-induced unheated milk gels

To investigate a possible non-specific effect of the reactive thiol group blocker NEM on the mechanical properties of acidified milk gels, non-heated reconstituted skim milk with and without NEM was compared. Reactive thiol groups were not present in unheated milk, and therefore specific effects of NEM can be excluded. The mechanical properties of the gels were monitored using both non-destructive, dynamic measurements within the linear region and destructive large deformation measurements. Figures 7.4 and 7.5 clearly show that for the acidified gels of both reconstituted and fresh skim milk without heat treatment there were no non-specific effects of NEM on mechanical properties, since both the cure curve (development of the storage modulus with time)(Figure 7.4) and the force distance-curve (Figure 7.5) proceed identically with and without the addition of NEM.

Effect of whey protein denaturation on mechanical properties

As already mentioned, heat treatment of milk results in the denaturation of whey protein in whey protein aggregates and in whey

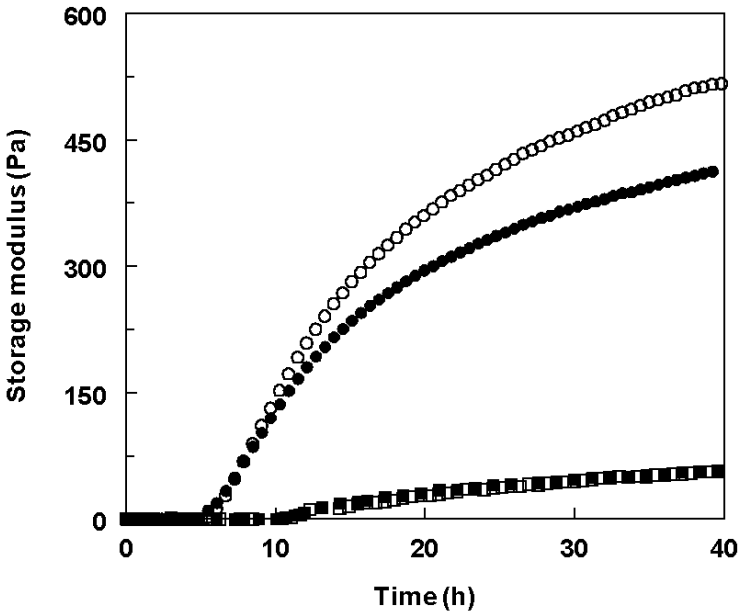

Figure 7.4: Development of the storage modulus with time for acidified non-heated (squares) and heated (circles) reconstituted skim milk in the absence (open symbols) and presence of NEM (closed symbols).

protein based coating of the casein micelles. To study the effect of this denaturation on the gel strength of acid milk gels without the interference of the additional formation of disulfide bonds during acidification, free thiol groups present after heat treatment were blocked by the application of NEM. The mechanical properties of the acidified milk gels were monitored using both non-destructive, dynamic measurements and destructive large deformation measurements. A gelation time of 11 and 5.5 hours was observed for unheated milk (Figure 7.4) and heated reconstituted skim milk treated with NEM (Figure 7.4), respectively, which was followed by a steady increase of the storage modulus, G', with time (Figure 7.4). In these measurements G' represents the elastic shear modulus of a gel. After 40 hours of acidification, heated reconstituted skim milk treated with NEM reached an approximately 7 times higher G' value than unheated reconstituted skim milk. Figure 7.5 shows the results of a large deformation study on acidified gels of unheated milk (open squares) and heated reconstituted skim milk with NEM (closed circles). Although gel hardness, expressed as the force (g) at the maximum of the force-distance curve (3), did not differ for NEM-treated heated milk and unheated milk, the slope of the force-distance curve of unheated and heated milk clearly differed. This slope relates to the elasticity of the gel. Qualitatively this agrees with

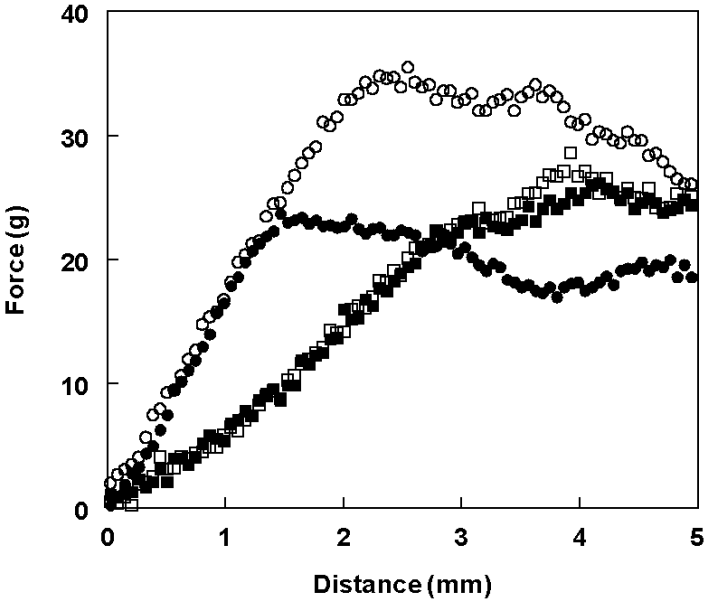

Figure 7.5: Effect of thiol-blocking on the large deformation properties of unheated (squares) and heated (circles) reconstituted skim milk in the absence (open symbols) and presence of NEM (closed symbols) after 24 hours of acidification.

the differences in elastic behaviour observed in the small deformation experiments.

The observed decrease in gelation time of NEM-treated heated milk compared to unheated milk is thought to be due to the whey protein coating of the casein micelles. The coating caused a change in the pI of the casein micelles from the pI of casein (4.6) towards that of whey protein (5.2). This has a clear effect on the gelation pH (13, 15, 21, 34) and subsequently on the gelation time as the same amount of GDL is applied. The elastic modulus of the gel clearly increases due to whey protein denaturation as the gels reach higher G'-values and have a steeper slope in the force-distance curves. The amount of additional protein available for structuring after heat treatment (approximately 20% of the total protein) is far too small to explain this increase. In electron microscopy pictures the formation of appendages on the casein micelles during heat treatment could be observed and it is generally believed that these interfere with the aggregation of the casein micelles and increase the interactions between the casein micelles (8, 13, 23), which explains the increase in elasticity of the gels. As the formation of disulfide bridges between the particles during the gel state was prevented by the NEM treatment in both cases only physical interactions (electrostatic, hydrophobic, hydrogen bonds) will occur, which finds expression in

the identical gel hardness obtained in the large deformation experiments. In conclusion whey protein denaturation clearly enhances elastic properties of acid milk gels.

Effect of disulfide bridge formation on the gel strength during acid-induced gelation

Figure 7.4 shows that the cure-curves of the non-treated (open circles) and NEM-treated (closed circles) heated reconstituted skim milk are identical at the start, and show a steady increase with time. After 10 hours of acidification a difference in G'-value is observed and increases with time. After 48 hours of acidification at ambient temperature the non-treated milk reaches a 20% higher G' value. With large deformation experiments a 30% increase in gel hardness was observed after 24 hours of acidification, while the slope was apparently not affected (Figure 7.5). With heated fresh skim milk a significant higher gel hardness was determined (50 g), than with heated reconstituted skim milk (35 g). Both NEM treated milks had a gel hardness of 23 g (graph not shown). Confocal scanning laser microscopy was applied to the different acid milk gels to observe potential differences in the micro structure. Neither the heat treatment nor the modification of the thiol groups were found to have an effect on the microstructure at a micrometer length scale (results not shown).

Comparison of heated milk with and without NEM treatment revealed the clear contribution of disulfide bonds to both the storage modulus and the gel hardness of acid induced milk gels. The increase in the elasticity of the gels (20%) is in agreement with the results of Lucey et al (21), who found a similar increase. The slope for acidified heated milk was hardly affected by the NEM treatment, indicating that the storage modulus is a more sensitive parameter to study the elastic behaviour. However, the gel hardness seems to be a very sensitive parameter to study disulfide bridge formation under acidic conditions. The experiments with fresh skim milk demonstrated that a higher number of thiol groups also resulted in a higher gel hardness. The amount of free thiol groups after heating correlates well with the gel hardness.

Effect of time on gel hardness

Figure 7.6 shows the development of the gel hardness with time for heated fresh skim milk at ambient temperature. The NEM-treated milk reaches a plateau value of less than 30 g after approximately 30 hours of acidification. The non-treated milk reaches a significantly higher gel hardness value than the NEM-treated milk. After 35 hours of acidification a value of 60 g is reached and the hardness steadily increases during further incubation to 90 g after 75 h. At this point there is still no levelling off.

Since in NEM-treated milk the ability to form disulfide cross-links is absent during acidification, the gel hardness-time curve represents the formation of a protein network built up of particles connected via physical interactions. The time needed to reach a plateau value seemed to correspond with the time needed to completely hydrolyse GDL, i.e. to reach the final pH of around 4.6. From that point on the environmental conditions will be constant and no further increase of gel hardness was observed. This would indicate that no further structural rearrangements take place that have an influence on the gel hardness. The additional contribution of covalent disulfide bonds between the particles is clearly seen on top of the formation of a physically linked protein network in the absence of

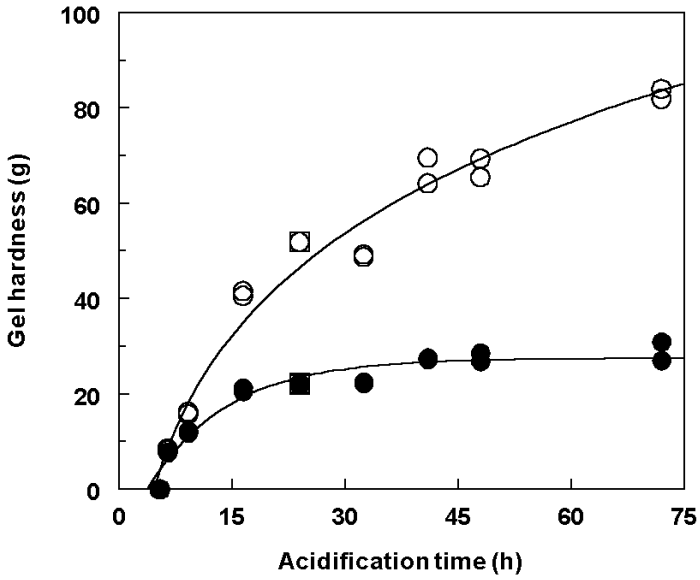

Figure 7.6: Development of the gel hardness with time for heated fresh skim milk in the absence (open symbols) and presence (closed symbols) of NEM. The lines are drawn to guide the eye. The squares mark the gel hardness after 24 hours of acidification of which the complete force-distance curves are shown in Figure 7.5.

NEM. This process is time dependent and proceeds steadily after the final pH is reached. Since it occurs on a time-scale of hours it is relevant for the textural properties of yogurt.

Formation of disulfide linked structures during gelation

SDS-agarose gel electrophoresis

The SDS-agarose gel electrophoresis was shown to be suitable to separate protein (aggregates) with a diameter ranging from 3 (monomeric protein) to approximately 250 nm. With this technique much larger protein structures can be determined than with classical polyacrylamide gel electrophoresis (PAGE) (1). Figure 7.7 shows that before gelation no effect is observed of the NEM treatment on the migration velocity of the protein bands (lanes 1 and 2). Since the agarose electrophoresis gels were run without stacking gel, diffuse bands were observed. A clear effect of the addition of NEM is observed after gelation. Only in the absence of NEM are slower migrating structures observed (bracket [a] in lane 3). In both cases addition of DTT resulted in the formation of a faster migrating protein band (arrow [c] in lanes 5 and 6). All samples contain a fast migrating protein band (arrow [d]); however the differences were observed in the slower migrating region. The protein band indicated by bracket [b] is only observed in heated milk.

Due to the presence of SDS non-covalent interactions are broken and only covalently bound structures are analysed. The casein micelles dissociate under these conditions resulting in a fast migrating protein band representing casein monomers and native whey proteins. The slower migrating band (bracket [b]) in lane 1 and 2 corresponds to a complex mixture consisting of disulfide linked casein monomers (oligomers of κ and α_{s2}-casein), complexes of cysteine-containing caseins and whey proteins and of whey protein aggregates (2, 4, 16, 24, 27, 28, 30, 32). This band is not observed after SDS-agarose gel electrophoresis of unheated milk (results not shown). After gelation disulfide cross-linked structures are formed with a lower migration velocity, which hardly enter the agarose gel. Blocking of thiol groups prevented formation of these larger structures. The reduction of disulfide bonds by DTT resulted in small sized protein material, probably corresponding to casein and whey protein monomers. In this case the disulfide linked structures formed during heat treatment between casein and whey proteins are also reduced. The application of both NEM and DTT clearly demonstrates

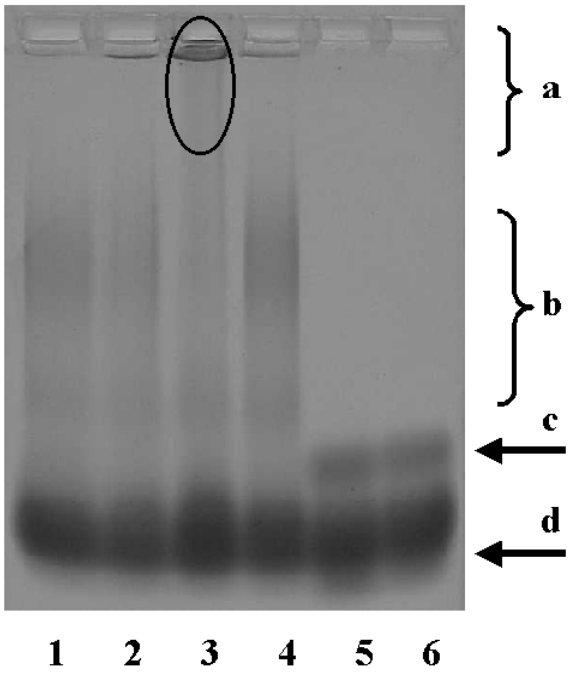

Figure 7.7: SDS agarose gel electrophoresis (0.7%) of heated reconstituted skim milk. All samples were dissolved in a SDS-containing buffer in order to break up physical interactions between the proteins. From left to right the lanes contain milk before gelation; -NEM (lane 1), + NEM (lane 2), after gelation; - NEM (lane 3), + NEM (lane 4), and after gelation + treatment with DTT after dissolving in SDS-buffer; - NEM (lane 5) and + NEM (lane 6). The arrows indicate the structures described in the results and discussion section (SDS-agarose gel electrophoresis). The circle highlights covalently linked proteins formed during acidification.

the formation of disulfide linked protein structures during acid induced gelation of heated milk.

Dynamic light scattering

A second method to observe the formation of disulfide linked structures during gelation is through application of enzymatically cross-linked reconstituted skim milk. Cross-linking of the casein micelles with transglutaminase prevents dissociation in the SDS-buffer needed to dissolve the acid milk gels (33). These modified casein micelles are used as a tool to determine disulfide linked structures by light scattering. To inactivate the enzyme transglutaminase, milk has to be heated for 25 min at 90°C. The number of exposed thiol groups decreased compared to reconstituted skim milk heated for 10 min (0.07 to 0.05 mM), but was similar to reconstituted skim milk heated for 25 min. The effect of NEM on the relative decrease in hardness of the gel prepared from transglutaminase-treated milk was comparable to normal treated

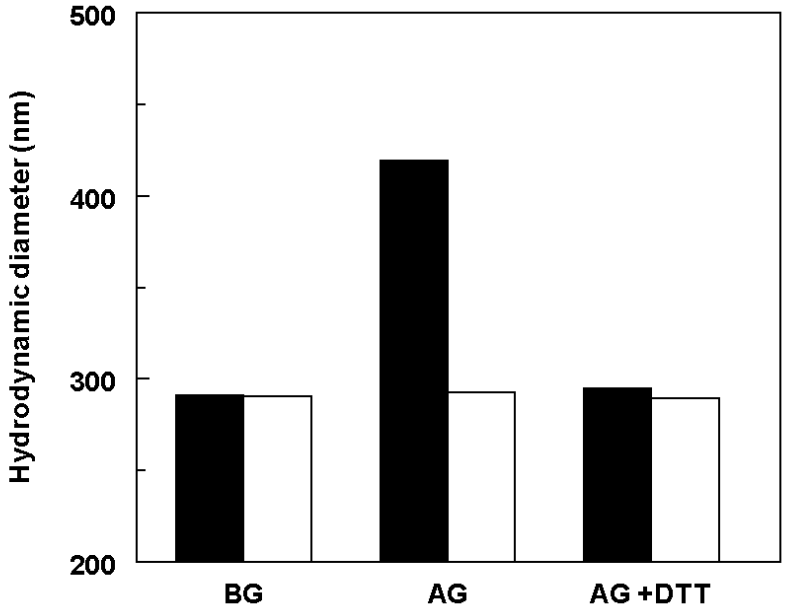

Figure 7.8: Size determination by light scattering of control (white bars) and blocked (black bars) samples of the intra-micellar cross-linked micelles, before gelation (BG), after gelation (AG) and after gelation + treatment with DTT (AG + DTT).

skim milk, indicating that the enzymatic modification does not affect the formation of free thiol groups nor the gel hardness after acidification.

Figure 7.8 shows the effect of gelation on the size of particles in milk before and after gelation in absence and presence of NEM. Before gelation, the modification of the thiol groups had no effect on the size of the particles in transglutaminase cross-linked skim milk. In both cases particles with a diameter of around 280 nm were determined. After gelation, only in the case of the heated milk sample without NEM treatment were particles with an increased diameter of 430 nm determined. The size of the particles could be decreased to its original value by the addition of DTT, an agent known to break up disulfide bonds.

The observed decrease in the number of thiol groups after prolonged heating, necessary to inactivate the enzyme transglutaminase, is probably due to oxidation of the thiol groups (12, 17, 18, 26), as in equally heated reconstituted skim milk the same decrease was determined. The size of transglutaminase cross-linked micelles observed is slightly larger than previously determined (19), which can be explained by the presence of SDS. Therefore, we can exclude the occurrence of inter-micellar cross-linking.

Ultracentrifugation experiments under non-dissociating conditions revealed that hardly any serum casein was released (33). SDS-agarose electrophoresis under dissociating conditions showed that the integrity of the micelle was retained (results not shown). A milk system was obtained which behaved identically to reconstituted skim milk, except that the casein micelles were no longer disrupted in SDS buffer. The light scattering results strengthen the previous results obtained by SDS-agarose gel electrophoresis, where disulfide linked structures formed during acid-induced gelation at 20°C were visualised.

General discussion

In our two stage approach we distinguish between heat treatment of milk and subsequent acid-induced gelation at 20°C. Whey protein denaturation as occurs in the first step is generally recognised as a very important parameter for the textural properties of yogurt and other acidified milk products. This is attributed to the altered physical properties of casein micelles due to heating and concomitant coating with whey proteins. The contribution of disulfide formation during the second step, i.e. acidification at ambient temperature, on the textural properties of acid-milk products is hardly recognised in the literature.

In this study we demonstrated that the time dependent formation of disulfide bonds during acidification contributes strongly to the mechanical properties. The cross-linking process continued even after complete hydrolysis of GDL, while the process levelled off at this point in the presence of NEM. The start of the gelation and the microstructure observed with CSLM were not affected by the modification of the thiol groups. Therefore, the increase in gel hardness and storage modulus was attributed to the additional disulfide cross-links formed, rather than to changes in the mechanism of aggregation and changes in the microstructure. Moreover, the gel hardness depended on the amount of reactive thiol groups present after heating the milk (heated fresh milk compared to heated reconstituted milk). As it is generally known that acidic conditions are not favourable for the formation of thiol-disulfide bond exchange reactions, it is surprising that these phenomena contribute to such a large extent to the texture of yogurt. Alting et al. (1) previously observed the importance of disulfide bridge formation in acid gels made of whey protein aggregates at ambient temperature. The cross-

linking was attributed to an increased protein concentration in the protein network due to acid-induced physical interactions between aggregates. As far as we know this is the first time that formation of disulfide cross-linked structures during acid-induced gelation of heated milk at ambient temperatures has been demonstrated. Since these processes take place at ambient temperature and on a time scale of hours this finding is highly relevant for the textural properties of acid-milk products, like yogurt. In fact this knowledge should allow us to control texture.

References

1. Alting, A.C., R.J. Hamer, C.G. de Kruif and R.W. Visschers. 2000. Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. J. Agric. Food Chem. 48: 5001.
2. Anema, S.G. and H. Klostermeyer. 1997. Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures above 100°C. J. Agric. Food Chem. 45: 1108.
3. Bourne, M.C. 1978. Texture profile analysis. Food Technology. 3: 62-66.
4. Corredig, M. and D.G. Dalgleish. 1996. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Res. Int. 29: 49.
5. Dannenberg, F. and H.G. Kessler. 1998. Reaction kinetics of the denaturation of whey proteins in milk. J. Food Sci. 53: 258.
6. Dannenberg, H. and H.G. Kessler. 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 1. Syneresis, Milchwissenschaft. 43: 632.
7. Dannenberg, F. and H.G. Kessler. 1988. Effect of denaturation of β-lactoglobulin on texture properties of set-style nonfat yoghurt. 2. Firmness and flow properties. Milchwissenschaft. 43: 700.
8. Davies, F.L., P.A. Shankar, B.E. Brooker and D.G. Hobbs. 1978. A heat-induced change in ultrastructure of milk and its effect on gel formation in yoghurt. J. Dairy Res. 45: 53.
9. Ellman, G.L. 1959.Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70.
10. Goddard, S.J. 1996. Effect of thiol reagents on the acid-heat-induced gelation of high-heat skim milk. J. Dairy Res. 63: 639.
11. Guincamp, M., G. Humbert and G. Linden. 1993. Determination of sulfydryl groups in milk using Ellman's procedure and clarifying reagent. J. Dairy Sci. 76: 2152.
12. Hashizume, K. and T. Sato. 1988. Gel-forming characteristics of milk proteins 1: Effect of heat treatment. J. Dairy Sci. 71: 1439.
13. Heertje, I, J. Visser and P. Smits. 1985. Structure formation in acid milk gels. Food Microstructure. 4: 267.
14. Hong, Y.H., K. Guthy and H. Klostermeyer. 1984. On the influence of SH-group in UHT milk during storage. Milchwissenschaft. 39: 5: 285.

15. Horne, D.S. and C.M. Davidson. 1993. Influence of heat treatment on gel formation in acidified milks. Protein and fat globule modification, in Proceedings of IDF Seminar. 267.
16. Jang, H.D. and H.E. Swaisgood. 1990. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. J. Dairy Sci. 73: 900.
17. Kirchmeier, O., M. El-Shobery and N.M. Kamal. 1984. Milcherhitzung und SH-gruppenentwicklung. Milchwissenschaft. 39: 12: 715.
18. Kirchmeier, O., N.M. Kamal and H. Klostermeyer. 1985. Milcherhitzung und SH-gruppenentwicklung II. Milchwissenschaft. 40: 12: 722.
19. de Kruif, C.G. 1997. Skim milk acidification. J. Colloid Interface Sci. 185: 19.
20. Lucey, J.A., M. Tamehana, H. Singh and P.A. Munro. 1998. Effect of interactions between denatured whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. J. Dairy Res. 65: 555.
21. Lucey, J.A., C. Tet Teo, P.A. Munro and H. Singh. 1997. Rheological properties at smal1 (dynamic) and large (yield) deformations of acid gels made from heated milk. J. Dairy Res. 64: 591.
22. Lucey, J.A., P.A. Munro and H. Singh. 1998. Rheological properties and microstructure of acid milk gels as affected by fat content and heat treatment. J. Food Sci. 63: 660.
23. Mottar, J. and A. Bassier. 1989. Effect of heat-induced association of whey proteins and casein micelles on yoghurt texture. J. Dairy Sci. 72: 2247.
24. Oldfield, D.J., H. Singh, M.W. Taylor and K.N. Pearce. 2000. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. Int. Dairy J. 10: 509.
25. Parnell-Clunies, E., Y. Kakuda, , J.M. deMan and F. Cazzola. 1988. Gelation profiles of yoghurt as affected by heat treatment of milk. J. Dairy Sci. 71: 582.
26. Pofahl, T.R. and D.G. Vakaleris. 1968. Effect of heat on sulfhydryl and disulfide groups of milk proteins as measured by the spectrofluorometric method. J. Dairy Sci. 51: 9: 1345.
27. Rasmussen, L.K., P. Højrup and T.E. Petersen. 1994. Disulfide arrangement in bovine caseins: localization of intrachain disulfide bridges in monomers of κ- and α_{s2}-casein from bovine milk. J. Dairy Res. 61: 485.
28. Rasmussen, L.K. and T.E. Petersen. 1991. Purification of disulfide-linked α_{s2}- and κ-casein from bovine milk. J. Dairy Res. 58: 187.
29. Schreiber, R. 2001. Heat-induced modifications in casein dispersions affecting their rennetability. Int. Dairy J. 11: 553.
30. Singh, H. 1993. Heat induced interactions of proteins in milk, Protein & Fat Globule Modifications-IDF Seminar. 191.
31. Tuinier, R. and C.G. de Kruif. 2002. Stability of casein micelles in milk. Accepted for publication in Journal of Chemical Physics.
32. Vasbinder, A.J., A.C. Alting and C.G. de Kruif. Heat-induced casein-whey protein interactions in milk, manuscript in preparation. Chapter 3 in this thesis.
33. Vasbinder, A.J. H.S. Rollema and C.G. de Kruif. Gelation mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase

cross-linked casein micelles, manuscript in preparation. Chapter 2 in this thesis.
34. Vasbinder, A.J., P.J.J.M. van Mil, A. Bot and C.G. de Kruif. 2001. Acid-induced gelation of heat treated milk studied by Diffusing Wave Spectroscopy. Colloids Surfaces B. 21: 245. Chapter 6 in this thesis.
35. Verheul, M., S.P.F.M. Roefs, and C.G. de Kruif. 1998. Kinetics of heat-induced aggregation of β-lactoglobulin. J. Agric. Food Chem. 46: 896.
36. van Vliet, T. and C.J.A.M. Keetels. 1995. Effect of preheating of milk o n the structure of acidified milk gels. Neth. Milk Dairy J. 49: 27.

Chapter 8: Practical Relevance

Practical relevance

In this thesis the casein-whey protein interactions occurring in heated milk are studied as a function of pH and temperature. The interactions of the milk proteins are related to the changed acid- and rennet-induced gelation properties. In chapter 4 a comprehensive model is presented of the distribution of whey proteins in aggregates and in the coating of the casein micelles. The model and the results obtained in chapter 3 to 7 are adding to the current knowledge required for controlling and optimising the production of dairy-derived products, like yoghurt, cheese, quark and dairy spreads. Additionally, chapter 2 provides information on the role of serum caseins in acid-induced gelation of unheated milk. The practical relevance of the findings in this thesis will be discussed briefly.

Chapter 2 shows that applying a sequence of decreasing the pH and increasing the temperature results in firmer gels which are less susceptible to syneresis than inducing gelation by first increasing the temperature and then decreasing the pH. The origin appears to be the distribution and the presence of serum caseins which may precipitate onto the casein micelles and therewith change the stability. Practically, understanding this mechanism opens possibilities to obtain firmer and more stable gels from the same starting material. Furthermore, it provides opportunities to pasteurise acidified milk products as slowly increasing the temperature of the liquid acidified milk will result in firm gels, which is in contrast to acid-milk gels which are destabilised easily upon warming up. This might reduce or completely prevent the use of artificial stabilisers.

Chapter 4 reveals that controlling casein-whey protein interactions by slight modifications in pH allows regulation of the gelation mechanism of acid and rennet-induced gels. Decreasing the pH before heat treatment increases the rennet-induced rate of flocculation of heated milk compared to that of milk heated at the natural pH of milk. This allows improvement of the rennetability of heated milk.

Chapter 7 demonstrates the increase in gel hardness due to disulfide bond formation during acid-induced gelation. The time-dependent formation of these disulfide bonds at ambient temperature allows increasing the gel hardness with the same starting material.

In conclusion this work shows that seemingly minor variations in milk treatment may lead to considerable changes in the properties of the end product. The model presented in chapter 4 accounts for the observed phenomena.

Summary

Milk

Milk and milk products have been consumed by people all over the world throughout recorded history. Milk is the secretion of the mammary glands of mammals. This work focuses on bovine milk produced by domesticated cows, which is referred to as milk throughout this thesis. Milk is a white opaque liquid with a Newtonian viscosity 1.5 times that of water. It is a mixed colloidal dispersion with remarkable stability. Milk can be boiled, frozen or dried and redispersed without any obvious changes in stability.

Skimmed milk consists of casein micelles of 0.1μm in radius, whey proteins of 0.003μm and numerous small molecules of less than 0.001μm. This thesis focuses on skimmed milk; the role of fat is not investigated. Casein micelles are association colloids consisting of different caseins, i.e. κ-, β-, α_{s1}- and α_{s2}-caseins, and clusters of calcium phosphate. Most of the calcium-sensitive caseins, i.e. β, α_{s1} and α_{s2}, are present in the interior of the micelle, while κ-casein is present at the exterior. The C-terminal part of κ-casein is very hydrophilic and has a considerable negative charge. This part of the molecule sticks out of the micelle into the surrounding medium as a flexible hair, forming a hairy brush at the colloidal surface and thereby stabilising the casein micelles. Most of the caseins in the micelles are adsorbed to calcium phosphate nanoclusters. Whey proteins are globular proteins, which remain in the serum upon acidification or renneting of the milk, while the casein micelles are precipitated under these conditions. The major whey proteins are β-lactoglobulin and α-lactalbumin. The numerous small particles present in milk are mainly lactose and salts. The main salts ions present in milk are potassium, sodium, calcium, magnesium, chloride and phosphate, of which calcium and phosphate are highly relevant for maintaining the integrity of the casein micelles.

Stability of casein micelles

Casein micelles in milk are stable at the pH of milk, i.e. 6.7, due to the stabilising hairy brush of κ-casein. Renneting, pH, salt, ethanol and other additives affect the κ-casein molecules and are therefore able to destabilise the micelle, causing aggregation and gelation. This thesis focuses on effects of heating, acidification and

renneting, as these processes are relevant to the dairy industry for yoghurt and cheese production.

Using the food grade chemical compound glucono-δ-lactone (GDL) allows controlled acidification without the possible interference of microorganisms. Upon slow acidification, hydrophobic interactions start to dominate over electrostatic repulsions as the pH where casein micelles tend to neutrality is approached. The brush of κ-casein molecules starts to collapse and the micelles aggregate and finally form a gel. Temperature and pH also affect the equilibrium of calcium and casein present in the micelle and in the serum. Decreasing pH and/or temperature causes a release of casein and calcium into the serum. In chapter 2 the mechanism of gelation as a function of pH and temperature is studied. Gelation was induced in two ways, i.e. by acidification of milk at temperatures ranging from 20 to 50°C (route T-pH) or by slowly increasing the temperature of acidified milk (pH 5.0 and higher; route pH-T). Via the pH-T route gelation occurs at higher pH and lower temperatures and the gels formed are stronger and show less syneresis than when gelation is induced via the T-pH route. Using modified casein micelles, which are no longer able to release serum caseins, demonstrate the crucial role of serum caseins. This phenomenon is schematically depicted in a model.

Upon renneting, the κ-casein molecule is cleaved into two fragments, i.e. casein macropeptide (CMP) and para-κ-casein. CMP is released into the serum, while para-κ-casein remains at the surface of casein micelles. However, para-κ-casein has lost the stabilising property of κ-casein and therefore renneting of milk causes aggregation and gel formation of the casein micelles.

Casein-whey protein interactions in heated milk

Heat treatment of milk at temperatures higher than 65°C causes major changes in the whey protein fraction and induces interactions between the whey proteins and the casein micelles. Due to heat treatment β-lactoglobulin unfolds and a reactive thiol group (–SH) is exposed. Reactive thiol groups can form disulfide links (S-S) with other exposed thiol groups (-SH SH-) and through disulfide bridge exchange reactions (-SH S-S). This process resembles a polymerisation process, in which the unfolding step of β-lactoglobulin represents the initiation. Both α-lactalbumin and κ-casein are involved in the polymerisation process through disulfide bridge

exchange reactions. Therefore, heating of milk results in a complex mixture of soluble whey protein aggregates composed of mainly β-lactoglobulin and α-lactalbumin and of casein micelles with a coating of associated whey proteins.

In chapters 3 and 4 this complex mixture of whey protein aggregates and whey protein coating of the casein micelles is unravelled. The fraction of whey proteins present in aggregates and coating is quantified and the homogeneity of the whey protein coating is studied. Heating milk at its natural pH (6.7) causes more denaturation at higher temperatures (temperature range 70 to 90°C). About 30% of the denatured whey proteins is present in soluble aggregates while the rest has associated with the casein micelle. The ratio of whey proteins present in aggregates and coating is constant at the different temperatures applied. The soluble whey protein aggregates have an estimated hydrodynamic diameter of 60 to 100 nm while casein micelles have a diameter ranging from 30 to 600 nm.

Changing the pH prior to heat treatment in the range of 6.3 to 6.9 causes a shift in the ratio of whey proteins present in aggregates and coating. Heating at higher pH leads to more soluble whey protein aggregates, while below pH 6.55 all whey proteins have associated with the casein micelle. Heating at pH 6.55 causes a rather homogeneous coating where most of the κ-casein molecules are involved while heating at lower pH values causes a more inhomogeneous coating with larger clusters of whey proteins leaving many unreacted κ-casein molecules. The pH-dependent denaturation behaviour of whey proteins and casein micelles is schematically depicted in a model (chapter 4) and can be considered as a main result of this thesis.

Renneting of heated milk

Heat treatment completely changes the rennet-induced gelation properties of milk. Heated milk has very poor clotting properties as is known to the cheese maker. In chapter 5 the effect of heating on the enzymatic cleavage of κ-casein and on the subsequent clotting process of the casein micelles are systematically studied. Three causes are investigated, i.e. 1] reduced enzymatic cleavage due to whey protein association with the κ-casein molecules, 2] precipitation of serum calcium due to heat treatment, as a result of which the serum casein concentration is nolonger sufficient to induce

aggregation of the renneted casein micelles, 3] stabilisation of the renneted casein micelles due to the charge of the associated whey proteins. It turns out that heating inhibits the enzymatic hydrolysis slightly; however the inhibition is not sufficient to cause the impaired clotting properties. Comparing five different methods to study enzymatic hydrolysis reveals that two methods frequently used in the literature are inappropriate. Calcium phosphate precipitation neither affects the enzymatic hydrolysis nor the clotting properties of heated milk. The final conclusion is that only the stabilising charge of the associated whey proteins is responsible for the impaired clotting properties of heated milk.

In chapter 4 the clotting properties of milk heated at pH 6.3 to 6.9 are investigated. It appears that although association of the whey proteins with the casein micelles causes the impaired clotting properties of heated milk, the homogeneity of coating is far more important than the absolute amount of whey proteins associated with the casein micelle.

Acidification of heated milk

The acid-induced gelation properties of milk are changed completely by heat treatment. Heating of the milk causes a shift of the pH at which gelation starts to higher pH values. In chapter 6 a direct evidence is given for the generally held opinion that denaturation of whey proteins causes this shift. Additionally, it is investigated which of the whey proteins is responsible for this shift. It appears that denaturation of β-lactoglobulin causes the entire shift in the onset of gelation, while α-lactalbumin does not play a(n) (additional) role in this process.

In chapters 3, 4 and 8 the contribution of whey protein aggregates and whey protein coating to the shift in gelation pH is studied. It turns out that aggregates change the gelation pH in a similar way as coating of the casein micelles with whey proteins, even though casein micelles and aggregates are not linked at pH 6.7. Low concentrations of aggregates are more effective in changing the gelation pH than low concentrations of whey proteins coating the casein micelles.

Disulfide bridge formation in acid-induced gels

The final acid-induced gels formed from heated milk are much firmer than gels made from unheated milk. In chapter 7 the contribution of denatured whey proteins and the formation of disulfide bridges during the gel state is investigated. It turns out that denatured whey proteins mainly change the elastic properties of the milk gel, while formation of disulfide bonds clearly increases the gel hardness. Formation of disulfide bonds is a time-dependent process which at ambient temperature causes a steady increase in gel hardness over time.

Conclusions

In conclusion, the research described in this thesis has extended the understanding of casein-whey protein interactions in heated milk and the relevance of these interactions for the gelation mechanism and final gel characteristics of acid- and rennet-induced gels. It has resulted in a detailed model of the interaction of whey proteins with casein micelles during heat treatment. The whey proteins form soluble aggregates but also associate to the surface of the micelles. The coating of the micelles is a sensitive function of the pH at which heat treatment is performed and is stronger at lower pH. Controlling the coating of the micelles and the formation of aggregates opens opportunities to regulate the gel strength of acid-milk gels and to improve the clotting properties of rennet-induced gels (curd). Both processes are highly relevant in dairy practice for controlling the quality of and developing new yoghurt and cheese-like products.

Samenvatting

Melk

Sinds mensenheugenis worden melk en melk producten door mensen over de hele wereld gegeten. Melk is een uitscheidings-product van zoogdieren. Dit proefschrift richt zich op melk van gedomesticeerde koeien. Dit wordt in de rest van dit proefschrift simpelweg als melk aangeduid. Melk is een witte, opake vloeistof met een viscositeit 1.5 keer die van water. Het is een zeer stabiele colloïdale oplossing, die kan worden gekookt, bevroren, of gedroogd en opnieuw kan worden opgelost zonder opmerkelijke veranderingen in de stabiliteit.

Magere melk bestaat uit caseïne micellen, wei-eiwitten en talrijke kleine moleculen met respectievelijke diameters van 0.1 μm, 0.003 μm en 0.001 μm en kleiner. In dit proefschrift wordt uitsluitend magere melk onderzocht, de rol van vet wordt daardoor volledig buiten beschouwing gelaten. Caseïne micellen zijn associatie colloïden die bestaan uit diverse caseïnes , te weten κ-, β-, α_{s1}- en α_{s2}-caseïne, en clusters van calcium fosfaat. β-, α_{s1}- en α_{s2}-caseïne behoren tot de groep van calcium gevoelige caseïnes die zich voornamelijk binnen in een caseïne micel bevinden. Het κ-caseïne is ongevoelig voor neerslaan door calcium en bevindt zich aan de buitenkant van de micel. Het C-terminale deel van κ-caseïne is zeer hydrofiel en heeft een negatieve lading. Dit gedeelte van het molecuul steekt uit de micel als een flexibele haar. Tezamen vormen deze haren een borstel die zorgt voor de stabiele eigenschappen van een caseïne micel. De caseïnes in een caseïne micel zijn grotendeels geadsorbeerd aan calcium fosfaat clusters. Wei-eiwitten zijn eiwitten die in oplossing blijven als de caseïne micellen coaguleren onder invloed van zuur of stremmen, zoals gebeurt bij de yoghurt of kaas bereiding. De belangrijkste wei-eiwitten in melk zijn β-lactoglobuline en α-lactalbumine. De talrijke kleine moleculen in melk zijn met name zouten en lactose. De belangrijkste zout ionen zijn kalium, natrium, magnesium, chloride, calcium en fosfaat. Calcium en fosfaat zijn cruciaal voor het handhaven van de integriteit van de caseïne micel door hun rol in de calcium fosfaat clusters.

Stabiliteit van caseïne micellen

Door de stabiliserende borstel van κ-caseïne zijn caseïne micellen stabiel bij de pH van melk, deze is 6.7. Door processtappen zoals stremmen, verzuren, toevoegen van zouten, alcohol of andere toevoegingen wordt de κ-caseïne beïnvloed. De borstel verliest zijn stabiliserende werking waardoor aggregatie en gelering plaatsvindt. In dit proefschrift richten we ons op verhitten, verzuren en stremmen, deze processtappen zijn relevant voor de bereiding van yoghurt en kaas.

Door gebruik te maken van een chemische component genaamd glucono-delta-lacton (GDL) is het mogelijk melk te verzuren op een manier die sterk lijkt op de natuurlijke verzuring van micro-organismen. Het grote voordeel is dat storende factoren veroorzaakt door de micro-organismen zelf voorkomen worden waardoor het gedrag van de eiwitten beter bestudeerd kan worden. Tijdens het langzaam verzuren van melk verliezen de eiwitten hun lading waardoor hydrofobe attracties, de elektrostatische repulsie gaan overheersen. De stabiliserende laag van κ-caseïnes verliest de repulsie waardoor de micellen kunnen aggregeren en uiteindelijk een gel vormen. Verlaging van de pH en / of temperatuur veroorzaakt een verschuiving van caseïne en calcium in de micel naar de oplossing, ook wel serum genoemd. In hoofdstuk 2 wordt het mechanisme van gelering als functie van pH en temperatuur bestudeert. Gel vorming wordt geïnduceerd via twee routes, namelijk door verhogen van de temperatuur (20-50°C) en vervolgens verzuren (TpH-route) of door verzuren bij kamertemperatuur gevolgd door langzaam opwarmen (pH-T-route). Gelvorming via de pHT-route vindt plaats bij een hogere pH en een lagere temperatuur. Bovendien zijn de gelen sterker en hebben minder neiging om te synereren dan wanneer ze gevormd worden via de TpH-route. Door gebruik te maken van melk met gemodificeerde caseïne-micellen, waarbij alle caseïnes gebonden zijn aan de micel, laten we de cruciale rol zien die serum caseines hebben op de gelvorming. Dit fenomeen is grafisch weergegeven in een model.

Stremmen van caseïne-micellen zorgt voor splitsing van κ-caseïne in 2 fragmenten, namelijk casein macro peptide (CMP) en para-κ-caseïne. CMP verdwijnt na afsplitsen in het serum, terwijl para-κ-caseïne gebonden blijft aan de micel. Echter para-κ-caseïne heeft niet de stabiliserende werking die κ-caseïne heeft waardoor

stremming zorgt voor aggregatie van de micellen en uiteindelijk resulteert in gel vorming

Caseïne – wei-eiwit interacties in verhitte melk

Verhitten van melk bij temperaturen hoger dan 65°C veroorzaakt interactie van caseïnes en wei-eiwitten. Dit wordt veroorzaakt door het ontvouwen van β-lactoglobuline waardoor een reactieve thiol groep (-SH) vrij komt. Deze reactive groep kan zowel met andere thiol groepen reageren als met disulfide bruggen (-S-S-). Dit laatste veroorzaakt openbreken van disulfide bruggen en vorming van deze bruggen op andere plekken. Belangrijke eiwitten in melk die een disulfide brug hebben zijn α-lactalbumine en κ-caseïne. Hierdoor ontstaat een complexe reactie waarin β-lactoglobuline kan reageren met α-lactalbumine en / of met zichzelf tot oplosbare wei-eiwit aggregaten en daarnaast met κ-caseïne kan reageren en zo een wei-eiwit coating op het oppervlak van de micel veroorzaakt. In al deze gevallen is β-lactoglobuline de initiator van het proces. Wei-eiwit dat met andere eiwitten heeft gereageerd wordt ook wel gedenatureerd genoemd.

In hoofdstuk 3 is dit complexe mengsel van caseines en wei-eiwitten ontrafeld. De melk wordt gefractioneerd in een deel met ongereageerde wei-eiwitten, een fractie met oplosbare gereageerde wei-eiwitten en een fractie van wei-eiwitten die met caseïne micellen hebben gereageerd. Daarnaast is onderzocht hoe homogeen de caseïne micel met wei-eiwit is bedekt. Bij de natuurlijke pH van melk (6.7) reageert ongeveer 30% van de gedenatureerde wei-eiwitten tot oplosbare aggregaten, de rest reageert met de caseïne micel. Hogere temperaturen zorgen voor meer denaturatie maar de percentages wei-eiwitten die respectievelijk met de caseïne micel reageren of tot oplosbare aggregaten wordt hier niet door beïnvloed. De grootte van de oplosbare aggregaten wordt geschat op 60 tot 100 nm, ter vergelijking een micel heeft een grootte variërend van 30 tot 600nm.

Veranderen van de pH van melk voor verhitten (6.3 tot 6.9) veroorzaakt een verschuiving in de ratio van wei-eiwitten aanwezig in aggregaten en op de micel. Verhitten bij een pH hoger dan 6.7 leidt tot meer wei-eiwit in oplosbare aggregaten, terwijl verhitten bij lagere pH meer coating van de micel geeft. Bij pH 6.55 wordt een homogene wei-eiwit coating op de micel gevormd, terwijl bij nog lagere pH deze inhomogener wordt. Door deze inhomogene coating

blijft een deel van de κ-caseïne moleculen ongereageerd. Het pH-afhankelijke denaturatie gedrag van wei-eiwitten in melk is schematisch weergegeven in een model (hoofdstuk 4) and wordt gezien als het belangrijkste resultaat van dit proefschrift.

Stremmen van verhitte melk

Verhitten van melk leidt tot een zeer matig stremgedrag van de melk, dat wil zeggen verminderde vlokking en gelering. In hoofdstuk 5 is het effect van verhitten op de enzymatische splitsing van κ-caseïne en op het vlokproces systematisch onderzocht. Drie mogelijke oorzaken zijn: 1] minder enzymatische splitsing door interactie van de wei-eiwitten met de κ-caseine, 2] verminderde vlokking door verlaging van de hoeveelheid calcium in oplossing door hitte-geïnduceerde precipitatie, 3] stabilisatie van de gestremde caseïne micel door de lading van de geassocieerde wei-eiwitten. Het blijkt dat verhitten de enzymatische hydrolyse iets remt, maar dit is niet voldoende om de slechte gelering te verklaren. Wel blijkt uit deze experimenten dat twee methodes die frequent in de literatuur worden gebruikt niet selectief genoeg zijn. Calcium fosfaat precipitatie heeft noch effect op de enzymatische hydrolyse noch op de vlokking. De uiteindelijke conclusie is dat de lading van de geassocieerde wei-eiwitten de oorzaak is van de verminderde vlokking.

In hoofdstuk 4 worden de strem eigenschappen van melk verhit bij diverse pH's (6.3-6.9) bestudeerd. Het blijkt dat behalve de hoeveelheid wei-eiwit op de micel met name de homogeniteit van de wei-eiwit coating een heel belangrijke factor is voor de mate van vlokking.

Verzuren van verhitte melk

De zuur-geïnduceerde gel eigenschappen van melk worden compleet veranderd door verhitten. Verhitten van melk veroorzaakt een verschuiving naar hogere gel pH's. In hoofdstuk 6 wordt direct bewijs geleverd voor de algemeen gevestigde mening dat gedenatureerde wei-eiwitten verantwoordelijk zijn voor deze verschuiving. Verder is onderzocht welk wei-eiwit verantwoordelijk is voor de verschuiving. Het blijkt dat alleen β-lactoglobuline dit

veroorzaakt en dat α-lactalbumine geen (aanvullende) rol hierin speelt, terwijl het wel denatureert.

In de hoofdstukken 3, 4 en 8 is bekeken welke individuele bijdrage wei-eiwit aggregaten en wei-eiwit coating op de verschuiving in gel pH hebben. Het blijkt dat beide groepen bijdragen aan deze verschuiving, zelfs al hebben de aggregaten en de micellen bij pH 6.7 geen interactie. Lage concentraties van wei-eiwit in de vorm van aggregaten blijken de pH van gelering effectiever te beïnvloeden dan wanneer eenzelfde hoeveelheid eiwit met de micel is geassocieerd.

Disulfide brug vorming in zuur-geïnduceerde gelen.

De zuur-geïnduceerde gelen gevormd van verhitte melk zijn veel steviger dan die gevormd uit onverhitte melk. Hoofdstuk 7 laat het effect op de gel sterkte van gedenatureerde wei-eiwitten en van disulfide brug vorming tijdens de gel fase zien. Het blijkt dat gedenatureerde wei-eiwitten met name de elastische gel eigenschappen van de melk gel bepalen, terwijl disulfide bruggen de gel hardheid verhogen. Vorming van disulfide bruggen is een tijdafhankelijk proces waardoor bij kamertemperatuur een geleidelijk toename van de gel hardheid in de tijd te zien is.

Conclusies

Het onderzoek beschreven in dit proefschrift draagt bij aan een beter begrip van caseine – wei-eiwit interacties in verhitte melk en de relevantie van deze interacties voor de gelering en de eigenschappen van de uiteindelijk door verzuring of stemming gevormde gelen. Dit heeft geleid tot een gedetailleerd model van interacties tussen caseïne micellen en wei-eiwitten, waarin wei-eiwitten ofwel oplosbare aggregaten vormen ofwel met de micel reageren. De mate en homogeniteit van coating is zeer afhankelijk van de pH. Door de manier van wei-eiwit denaturatie te beïnvloeden, kan op natuurlijke wijze de gelsterkte van zure gelen gecontroleerd worden. Voor gestremde gelen kan deze kennis gebruikt worden om zo'n sterk mogelijk gel te verkrijgen uit verhitte melk. Deze beide processen kunnen zeer relevant zijn voor het ontwikkelen van nieuwe yoghurt en kaas-achtige producten.

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Astrid

Curriculum Vitae

Astrid Vasbinder is op 30 april 1974 geboren in Gouda. In 1992 heeft zij haar Gymnasium diploma behaald aan het Coornhert Gymnasium te Gouda. In datzelfde jaar begon zij met de studie Levensmiddelentechnologie aan de Landbouw Universiteit Wageningen, oriëntatie levensmiddelenchemie en –microbiologie. Deze studie werd in 1997 cum laude afgesloten met twee afstudeervakken en een stage. Deze werden uitgevoerd bij de vakgroep Levensmiddelenchemie in Wageningen, aan de Landbruks Universitet in Uppsala te Zweden en bij de afdeling Microbiologie van Unilever Research en Development in Vlaardingen. In juni 1997 kreeg zij bij Unilever een aanstelling als product development manager op de afdeling Culinary Products. In oktober 1998 begon zij als wetenschappelijk medewerker bij NIZO food research voor een periode van vier jaar, in welke tijd dit proefschrift tot stand is gekomen. Het onderzoek vond plaats in samenwerking met Unilever Research and Development als onderdeel van een BTS project. Het promotie onderzoek vond plaats onder begeleiding van Prof. Kees de Kruif, werkzaam bij NIZO food research te Ede en als hoogleraar verbonden met de afdeling Fysische en Colloid Chemie van de Universiteit van Utrecht.

List of publications

Vasbinder, A.J., P.J.J.M. van Mil, A. Bot and C.G. de Kruif. 2001. Acid-induced gelation of heat treated milk studied by Diffusing Wave Spectroscopy. Colloids and Surfaces B, 21: 245

Vasbinder, A.J., H.S. Rollema, A. Bot and C.G. de Kruif. 2003. Gelation Mechanism of milk as influenced by temperature and pH; studied by the use of transglutaminase cross-linked casein micelles. Accepted for publication in the Journal of Dairy Science.

Vasbinder, A.J., A.C. Alting and C.G. de Kruif. 2003. Heat-induced casein-whey protein interactions in milk. Accepted for publication in Colloids and Surfaces B.

Vasbinder, A.J. 2003. Casein micelle whey protein interaction in heated milk. Industrial Proteins in Perspective. Progress in Biotechnology. Elsevier. In Press.

Vasbinder, A.J., H.S. Rollema and C.G. de Kruif. 2003. Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics. Accepted for publication in the Journal of Dairy Science

Vasbinder, A.J., A.C. Alting, R.W. Visschers and C.G. de Kruif. 2003. Texture of acid-milk gels: formation of disulfide cross-links during acidification. Accepted for publication in the International Dairy Journal.

Vasbinder, A.J. and C.G. de Kruif. Casein-whey protein interactions in milk heated at pH 6.3 – 6.9. Submitted for publication.

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